ABSTRACT

Title of Thesis:	SPRAY STRATEGIES AND SELECTION FOR FUNGICIDE RESISTANCE: FENHEXAMID RESISTANCE IN BOTRYTIS CINEREA AS A CASE STUDY
	Stephen Carl Boushell, Master of Science, 2023
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Fungicide resistance is a limiting factor in sustainable crop production. Despite the wide adoption of general resistance management strategies by growers, the recent rate of resistance development in important fungal pathogens is concerning. In this study, *Botrytis cinerea* and the high-risk fungicide fenhexamid were used to determine the effects of fungicide dose, tank mixture, and application timing on resistance selection across varied frequencies of resistance via both detached fruit assays and greenhouse trials. The results showed that application of doses lower than the fungicide label dose, mixture with the low-risk fungicide captan, and application post-infection seem to be the most effective management strategies in our experimental settings. In addition, even a small resistant *B. cinerea* population can lead to a dramatic reduction of disease control efficacy. Our findings were largely consistent with the recent modeling studies which favored the use of the lowest possible fungicide dose for improved resistance management.

SPRAY STRATEGIES AND SELECTION FOR FUNGICIDE RESISTANCE: FENHEXAMID RESISTANCE IN BOTRYTIS CINEREA AS A CASE STUDY

by

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Introduction

The recent rate of emergence of plant pathogens with resistance to a limited number of commonly used fungicides is unprecedented and is considered a serious challenge to the sustainability and security of crop production (Fisher et al., 2018). Selection for resistance in fungal pathogen populations is a direct outcome of fungicide applications and the rate of resistance emergence is determined by spray strategies, fungal genotype, and fungal reproductive cycle (Lucas et al., 2015). General resistance management strategies such as rotation and mixtures of fungicides have been widely implemented. However, limited lab experiments or trials have been conducted to document the effects of fungicide rates, spray timing, and mixing partners on resistance selection comprehensively (van den Bosch, Paveley, et al., 2014). Further, to the best of our knowledge, no efforts have been made to quantify the extent to which the efficacy of fungicides is compromised under varying frequencies of fungicide resistance. Understanding the efficacy of fungicides under varied resistance scenarios is key to the interpretation of resistance monitoring data and identification of effective fungicides.

Ideally, a fungicide dose recommended for a given diseases' management needs to reach its highest possible efficacy while selecting for resistance to the least extent. Greater disease control efficacy of fungicides is expected when applied at a higher rate to control entirely sensitive fungal populations (Berg et al., 2016). However, to the best of our knowledge, no previous studies have specifically tested the disease control efficacy of fungicide doses under

varied resistance scenarios, and thus guidance for dose rates in the presence of resistance is limited. Previous literature has indicated that the optimal dose for resistance management is dependent on the phase of resistance development in which the fungicide application is made (van den Bosch et al., 2011). The first stage of resistance development, emergence, is the period in which a resistance-conferring mutation is not yet present in the fungal population. The second stage, selection, occurs after a resistance-conferring mutation is introduced into the fungal population. At this point, the continued use of the fungicide will increase the frequency of resistant fungal isolates in the population (van den Bosch et al., 2011). The selective pressure of chemical applications forms more favorable conditions for isolates with the resistance-conferring mutation than the other remaining sensitive isolates. Previous integrated pest management guidance has cautioned against the application of fungicides at lowered rates for proper resistance management (Beckerman et al., 2015). Despite this, there remains little consensus on higher or lower fungicide doses resulting in greater resistance selection. Field studies have observed greater resistance selection resulting from lowered fungicide dose applications (Ayer et al., 2020; Gubler et al., 1996; Steva, 1994). However, there have been other laboratory growth chamber, field, and mathematical modeling studies supporting the application of lower doses for lowered resistance selection (Genet et al., 2006; Metcalfe et al., 2000; van den Bosch et al., 2011; van den Bosch, Oliver, et al., 2014). In addition, a previous study testing the efficacy of a fungicide dose at half the rate of the labeled dose found the lowered application dose resulted in lowered resistance selection. However, the lower resistance selection resulting from the halved application rate was accompanied by decreased disease control (Dooley et al., 2016). Further complicating the situation, some studies have found the dose to have no significant impact on the selection of fungicide resistance (Burnett & Zziwa, 1997; Pijls & Shaw, 1997).

Another major focus of this research is to evaluate the merit of resistance management strategies pertaining to the mixture of high-risk fungicides and low-risk (multi-site) fungicides. Unlike high-risk fungicides, low-risk fungicides are much less prone to resistance development in fungal populations, but they are regarded as less effective and less safe in general (Adaskaveg et al., 2005). Mixing high-risk fungicides with low-risk fungicides may therefore provide insurance for disease control and improve resistance management (Hobbelen et al., 2013; Mikaberidze et al., 2014). Thus, it is generally advised to mix high-risk, single-site fungicides with low-risk, multi-site fungicides to limit resistance emergence and slow resistance selection. Previous studies have attempted to determine the efficacy of mixing fungicides to limit resistance selection and disease control. Previous field study has found mixture to result in lessened shifts towards resistance while effectively maintaining disease control (Forster & Staub, 1996; Oxley et al., 2008). Although, previous field work which did not observe a reduction in resistance selection resulting from the mixing with the multi-site fungicide, captan, exists (Northover, 1986). Mathematical modeling studies have found that a mixture of high and low-risk fungicides in appropriate proportions can prove effective at maintaining disease control of the target pathogen without significantly increasing selection for resistance (Mikaberidze et al., 2014). These fungicide mixture combinations have also been shown to potentially extend the effective lives of the single-site fungicides compared to applications of the single-site fungicide alone (Berg et al., 2016).

The timing of fungicide application plays a significant role in the effectiveness of the chemicals on disease control (Petit et al., 2010). Often fungicides are applied in a preventative, pre-application manner based on spray schedules to stop potential fungal outbreaks and field experimentation has shown greater disease control the earlier the fungicide application occurs

(Petit et al., 2010). Despite this, other previous field studies have shown fungicide applications initiated after disease detection or artificial inoculation to be as effective as preventative sprays (McGrath, 1996; Simpfendorfer, 2016). Importantly, the specific mode of action of the chemistries applied should be considered. Contact (multi-site) fungicides primarily provide protection on the surface of vulnerable plant tissue. In laboratory assays they have been shown to provide high toxicity for conidial germination inhibition, however, they are significantly less effective at limiting mycelial growth of *Botrytis* (Elad et al., 2007). Thus, current recommendations encourage their use only in pre-application manners given their mode of action is purely preventative, given their lack of systemic post-infection protection (Thind & Hollomon, 2018). Systemic fungicides have been shown to have a "kickback" effect when applied after fungal infection. As the plants can absorb and transport the fungicide, they incur greater, more uniform, protection despite poor spray coverage or delayed application timing (Klittich, 2014). Thus, a greater opportunity may exist for their successful use during disease outbreaks and instances of higher disease pressure. In addition, the accepted practice has advised against the use of fungicides in a curative, post-application manner for resistance management (Brent, 1995). It is reasoned that this practice may elicit greater resistance selection when a larger population of the pathogen is present and should be avoided for resistance management strategies (Beckerman et al., 2015). Despite the risks associated with post-infection fungicide sprays, previous studies have shown that delaying the first fungicide application on grapevines until the first day of disease onset could halve fungicide use for similar efficacy and decrease operator exposure (Chen et al., 2020). This chemical exposure remains a great concern regarding the long-term use of multi-site fungicides. Another study testing the effect of delayed initial fungicide application found effective disease management was achieved despite delaying the

first preventative fungicide application of the season (Caffi et al., 2010). However, to the best of our knowledge, no studies have tested the efficacy of delaying fungicide application timing for resistance or disease management against fungal populations with varied frequencies of resistance.

Botrytis gray mold, causal agent *Botrytis cinerea,* is a serious disease globally causing significant economic losses and remains one of the most important fungal diseases on various fruit, vegetable, and ornamental plants (Dean et al., 2012). The wide range of hosts impacted by the disease, more than 1400 plant species globally, underly the importance of effective disease management (Elad et al., 2016). Management of the pathogen relies heavily on regular fungicide applications throughout the growing season (Hu et al., 2016). As a polycyclic fungal pathogen, *B. cinerea* may develop fungicide resistance quickly without proper stewardship of effective active ingredients. *B. cinerea* is regarded as a model pathogen due to its proclivity for developing resistance to new fungicide classes and often to forming multi-fungicide resistance (Fan et al., 2017; Weber, 2011). With limited remaining effective fungicidal products labeled for botrytis control due to resistance development to many common FRAC groups, resistance management to the effective extant fungicide products currently available remains imperative. Resistance to the single-site fungicide fenhexamid (tradename Elevate 50WDG, FRAC 17) has been observed in *B. cinerea* populations in small fruit fields throughout the Mid-Atlantic at the frequency of 48% (Cosseboom & Hu, 2021). In contrast, captan-based fungicide products have remained important for disease control and resistance management for *Botrytis* disease outbreaks due to intrinsic difficulty in developing resistance to the multiple modes of action of the fungicide.

Often studies have examined the effect of dose, mixing, or application timing individually under a single frequency of resistance. The aim of this study is to understand the

effects of these potential resistance management strategies together across varied starting resistance scenarios to better understand their effectiveness at limiting resistance selection while managing disease effectively. As noted above, resistance to fenhexamid in *B. cinerea* populations is not uncommon and that the resistance frequency can vary between locations and seasons (Fernández-Ortuño et al., 2014). The first objective of this research is thus to determine the efficacy of fenhexamid and captan fungicide application methods for controlling *B. cinerea* populations with varying frequencies of resistance. It is hypothesized that the highest fungicide dose, application of the fungicide pre-infection, and addition of a low-risk fungicide are the most effective strategies for managing disease for entirely sensitive fungal populations as well as scenarios in which resistance is present. The second objective of this research is to determine the impact of high-risk fungicide dose, the involvement of low-risk fungicides, and the timing of fungicide applications on resistance selection. It is hypothesized that the lowest fungicide dose and addition of a low-risk fungicide are the most effective strategies for managing fungicide-resistant populations, regardless of application timing.

Materials and methods

Detached fruit assay organization

A series of four unique detached fruit assays (three generations) on organic table grapes were conducted and subsequently repeated to test the major factors outlined above. Across the detached fruit assays a total of ten fungicide treatments were tested, however not all fungicide treatments were included in all assays due to practical limitations. All other factors were consistent between all the detached fruit assays.

Table 1. Fungicide treatment outline across the four unique detached fruit assays.

		Fungicide Treatment[y]									
Detached Fruit Assay	**Resistance Scenario[x]**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Assay 1 (Pre-Application)	Scenario 1	X	X			X	X	X			X
	Scenario 2	X	X			X	X	X			X
	Scenario 3	X	X			X	X	X			X
	Scenario 4	X	X			X	X	X			X
Assay 2 (Pre-Application)	Scenario 1	X		X	X	X	X		X	X	X
	Scenario 2	X		X	X	X	X		X	X	X
	Scenario 3	X		X	X	X	X		X	X	X
	Scenario 4	X		X	X	X	X		X	X	X
Assay 3 (Post-Application)	Scenario 1	X	X			X	X	X			X
	Scenario 2	X	X			X	X	X			X
	Scenario 3	X	X			X	X	X			X
	Scenario 4	X	X			X	X	X			X
Assay 4 (Post-Application)	Scenario 1	X		X	X	X	X		X	X	X
	Scenario 2	X		X	X	X	X		X	X	X
	Scenario 3	X		X	X	X	X		X	X	X
	Scenario 4	X		X	X	X	X		X	X	X

[x] Percentage of resistant spores in starting inoculum for scenarios 1-4 (0, 3, 10, and 30%).
[y] Fungicide treatment concentrations and mixing partner presence are outlined in Table 2.

Isolate selection and media preparation

To ensure a good representation of resistant genotypes, four *Botrytis cinerea* isolates previously collected and identified as fenhexamid-resistant were selected and used in this study (Cosseboom & Hu, 2021; Hu et al., 2018). To confirm the phenotype of each isolate, cultures were grown on potato dextrose agar (PDA) and incubated at 22˚C in light conditions until sporulation. Spores were harvested from the cultures and plated on both malt extract agar (MEA) and malt extract agar amended with the discriminatory dose of 50 µg/mL fenhexamid previously described (Weber & Hahn, 2011). The plates were incubated at 22˚C in dark conditions to induce germination. After 18 hours the lengths of the germination tubes were measured on both MEA and MEA amended with 50 µg/mL fenhexamid. Isolates with germination tubes at least 50% the lengths on the amended media compared to the unamended media were classified as resistant.

Isolates with germination tubes were less than 10% of the lengths on the amended media compared to unamended media were classified as sensitive consistent with the stalled germination tubes of fenhexamid-sensitive isolates observed in Weber & Hahn (2011). Very few isolates had germination tubes between 10 - 50% of the lengths of the germination tubes on unamended media, which were excluded and not selected for use in the detached fruit assays.

Inoculum preparation

Spore suspensions were prepared from a combination of eight above-mentioned *B. cinerea* isolates. Autoclaved tap water was used to flood the plates of sporulating cultures. Tween 20 was added to the water to facilitate the collection of spores. The resulting spore suspensions from each of the sensitive isolates were mixed and diluted to a spore concentration of $1x10^5$ spores/mL (Dowling et al., 2016). This was repeated for the four resistant isolates. Then the spore suspensions composed entirely of sensitive and resistant isolates of equal spore concentrations were mixed to produce spore suspensions of 0%, 3%, 10%, and 30% resistant spores, which served as the starting inocula for the detached fruit assays.

Fungicide application on detached grape berries

Organic grapes were surface sterilized using a 10% sodium hypochlorite solution for two minutes, followed by rinsing in autoclaved deionized water for two minutes to remove residue. Then they were dried for two hours in an open biosafety cabinet. Fungicide treatments varied in the timing of their application relative to the artificial inoculation and subsequent infection. Grapes treated in a pre-application manner were sprayed with their corresponding fungicide

treatments immediately following surface sterilization and kept in the biosafety cabinet for 30 minutes until dry. In contrast, grapes treated in a post-application manner were inoculated with 15 µL of spore suspension directly after surface sterilization. The corresponding fungicide treatments were applied 48 hours later once disease symptoms were visible on most of the berries (Figure 1). In addition, fungicide treatments varied in concentration and the presence of a multi-site mixing partner. Concentrations of the single-site active ingredient included 1198 µg/mL, 839 µg/mL, 210 µg/mL, 25 µg/mL, and a 0 µg/mL control. The dose of 1198 µg/mL was determined from the application rate on the fungicide label. The additional lower doses of 839 µg/mL, 210 µg/mL, and 25 µg/mL were included to test whether doses less than that of the fungicide label were more effective at limiting resistance selection. The 4793 µg/mL concentration of the multi-site fungicide was determined by a medium rate on the fungicide label (Table 2).

Table 2. Outline of the active ingredient concentrations of the ten fungicide treatments in the detached fruit assay experiments.

Fungicide Treatment	Fenhexamid[x] Concentration (µg/mL)[z]	Captan[y] Concentration (µg/mL)[z]
1	1198	0
2	839	0
3	210	0
4	25	0
5	0	0
6	1198	4793
7	839	4793
8	210	4793
9	25	4793
10	0	4793

[x] Trade name Elevate 50 WDG
[y] Trade name Captan 80 WDG
[z] Fungicide doses were determined based on the application rate of 50 gallons of water per acre.

Inoculation and harvest

Each grape was gently stabbed via an autoclaved toothpick to create a wound site for inoculation with 15 μL of spore suspension. The grapes were incubated in a sterilized box with moistened paper towels to maintain a high relative humidity and induce fungal infection. After 48 hours the lids were removed from the boxes to allow for a decrease in humidity to promote sporulation on the infected grapes. After another five days, the diameter of the lesions was measured for each berry. The lesion measurements included both a vertical diameter measurement as well as a horizontal diameter measurement. The following day, spores were harvested from all berries from each replicate. The spores were harvested by manually scraping the surface of the berries with a moistened inoculation loop. A spore suspension was obtained from each replicate, which was subsequently plated on both MEA and MEA amended with 50 μg/mL fenhexamid to determine the ratio of the resistant phenotype. Then spore suspensions obtained from each replicate, which were used in the plating for fungicide resistance quantification, were then used to inoculate the next set of grapes and acted as the starting inoculum for the subsequent generation. This process of harvesting spore suspensions from the disease fruit to serve as the inocula for the next generation was conducted twice such that each detached fruit assay was continued for three successive generations (Figure 1).

Spore counting and phenotyping

The MEA plates spread with spore suspensions were incubated at 22°C under dark conditions to promote spore germination. After 18 hours the plates were removed, and the relative frequency of resistance was measured for each replicate. The phenotype of thirty spores was observed by analyzing three groups of ten spores on each plate. Consistent with the protocol

for isolate selection, spores on the fungicide amended plates were counted as resistant if their germination tubes were at least 50% the length of the average germination tube on their corresponding unamended plate. In contrast, spores were classified as sensitive if their germination tubes were less than 10% the length of the average germination tube on their corresponding unamended plate (Figure 2). Spores with unclear phenotypes and germination tubes between 10 - 50% of the average lengths of the germination tubes on unamended media were rare and excluded.

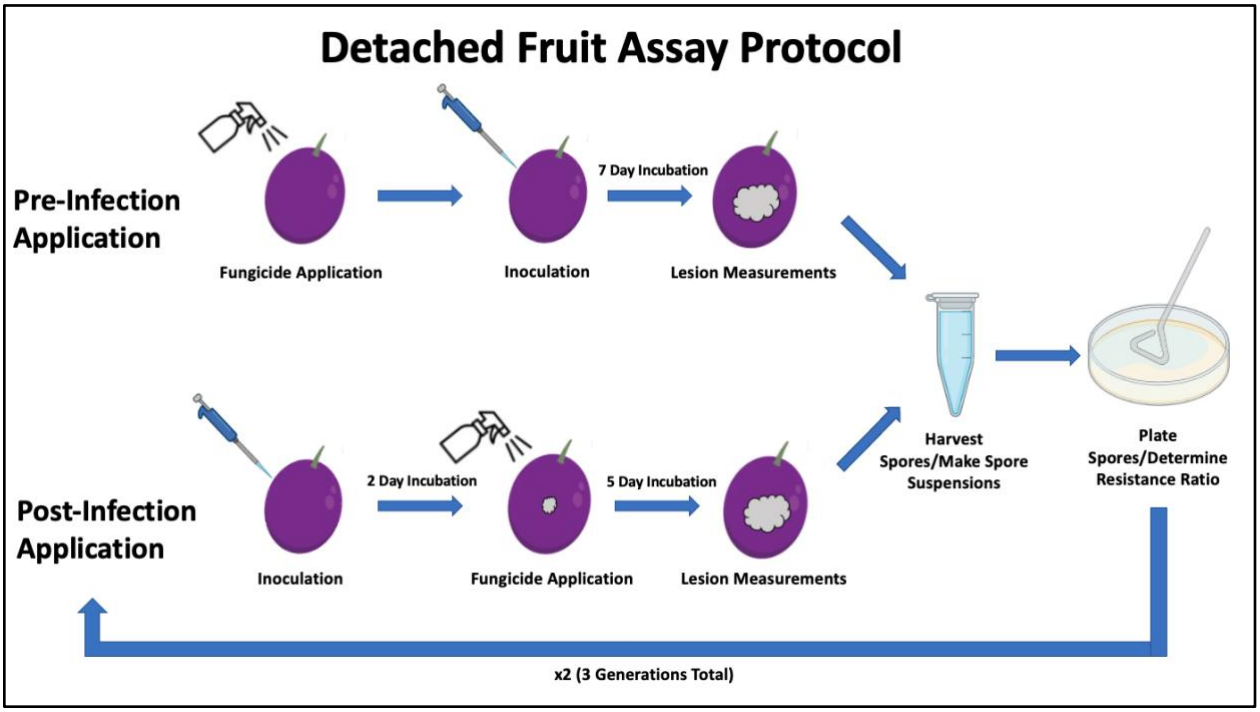

Figure 1. Protocol for the pre-application and post-application detached fruit assays. The figure shows the process for a single generation. Each detached fruit assay includes two additional generations not illustrated above, which are inoculated with the spore suspensions obtained from the previous generation's grapes.

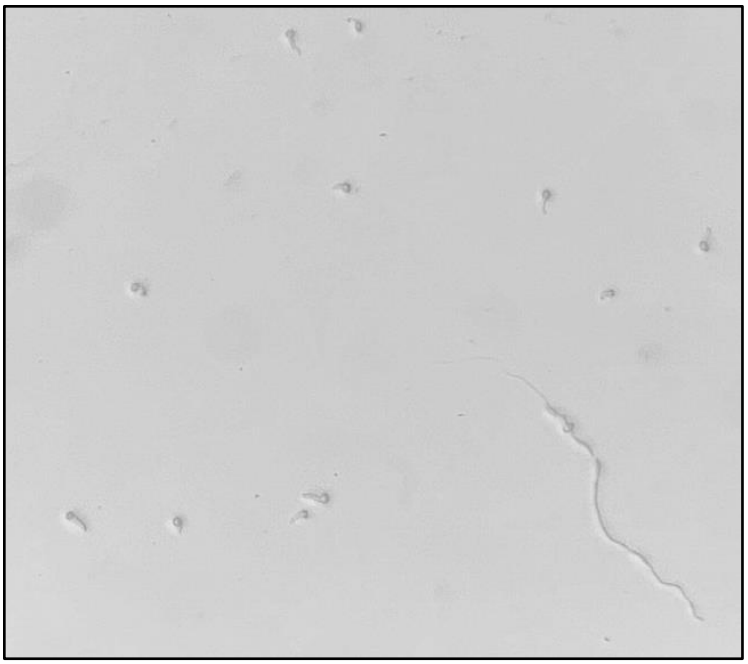

Figure 2. Examples of *Botrytis cinerea* spores on MEA plates amended with 50 µg/mL fenhexamid. Sensitive spores with short, stunted germination tubes are visible throughout the field of view. A single resistant spore with normal germination tube elongation consistent with the germination of spores on unamended MEA control plates is visible on the lower-right quadrant of the field of view.

Statistical analysis

Eight individual detached fruit assay datasets were combined. Disease lesion diameter measurements were taken over three generations through three individual rounds of inoculation and fungicide application. The lesion diameters were averaged across the three generations to quantify *Botrytis* disease severity. In addition, the ratio of fenhexamid resistance was averaged across the three generations to evaluate the resistance selection for each unique fungicide treatment. Using the JMP Pro 15 software, the average lesion diameter and average resistance rate data were tested for unequal variances to determine the appropriateness of combining datasets. The Brown-Forsythe test was used to compare the variance of the four unique detached fruit assays to their four respective repetitions. The four tests resulted in p-values of 0.43, 0.81, 0.31, and 0.28 for the average lesion diameter values. Testing the second response variable,

average resistance rate, resulted in p-values of 0.82, 0.08, 0.32, and 0.29. Further, the four unique detached fruit assay datasets once combined with their respective repetitions were combined across application timings through the comparison of the 95% confidence intervals of two fungicide treatments common throughout all detached fruit assays. Significant overlap existed in the 95% confidence intervals for these fungicide treatments indicating strong consistency in the average resistance rate and lesion diameter measurements across assay types. The successful combination of datasets allowed for the analysis of variance between the means of both response variables with the all-pairs Tukey-Kramer HSD test. To determine the effect of individual factors on both disease severity and resistance selection, fungicide treatments varying only in the factor of interest were compared. The results of all comparisons of a particular factor were compiled despite differences in the other factors that were tested simultaneously. Thus, herein the term "situations" is used to reflect the diversity of other factors that were blocked during the comparison of the factor of interest. As a result, the term may carry a unique meaning dependent on the individual factor of interest being discussed.

Results

Efficacy of fungicides under varied frequencies of resistance

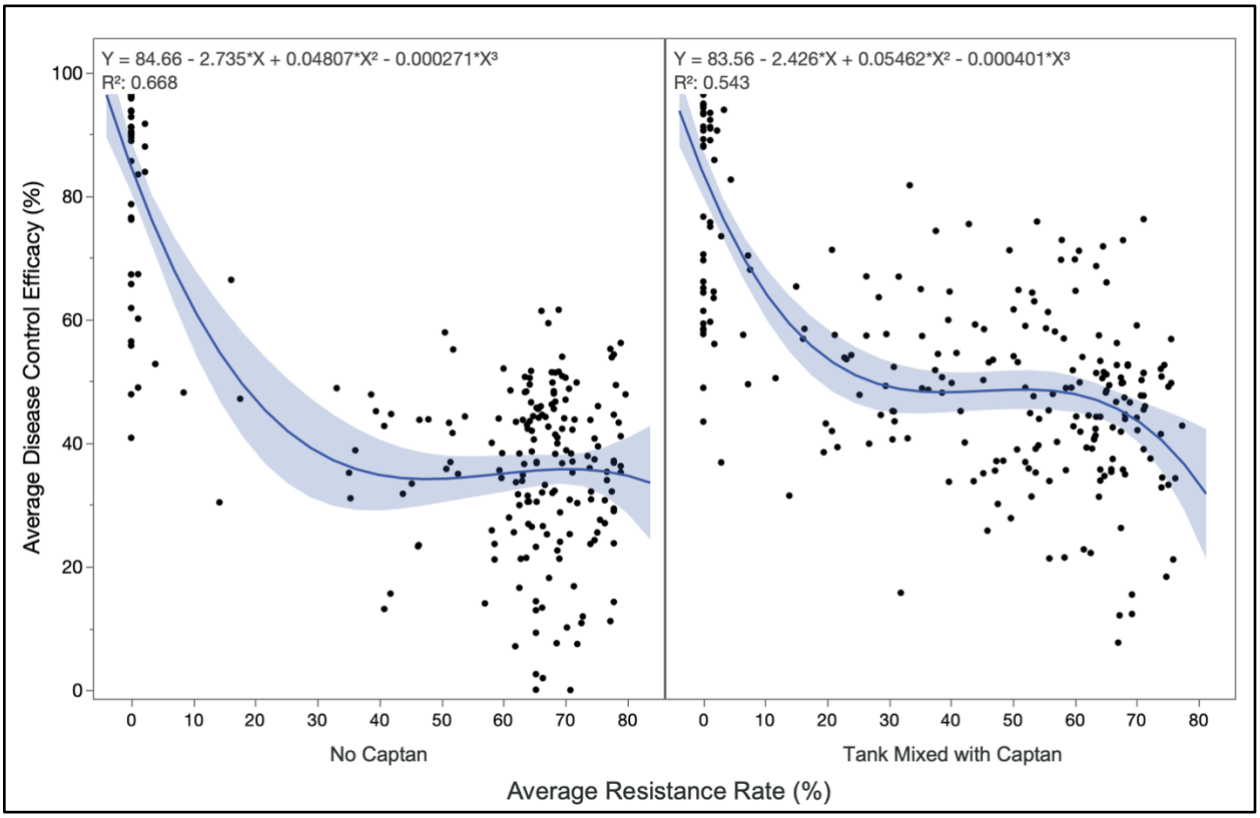

Figure 3. Fungicide efficacy under varied frequencies of resistance to Fenhexamid.

The efficacy of the single-site fungicide, fenhexamid, for controlling *Botrytis* was assessed under varied resistance scenarios. In Figure 3, a correlational analysis was conducted to understand the extent to which the disease control of the fungicide was lost as the rate of fungicide resistance in the inocula increased. This analysis was stratified based on the presence of the multi-site mixing partner, captan. Both fitted polynomial curves resulted in low p-values, <0.0001, and a significant positive cubic association between the average resistance rate of the inocula and the resulting average disease control efficacy of the fungicide treatments was observed for the analysis of variance. Further, the curves resulted in R^2 values of 0.668 and 0.543 indicating that approximately 66.8% and 54.3% of the total variability in average disease control

efficacy can be explained by its association with the average resistance rate for scenarios of no mixture and those with captan, respectively. Effective disease control was widely observed at rates of very low resistance (0-10%) as indicated by the high y-intercept values of the curves of 84.66% and 83.56%. Importantly, disease control efficacy decreased sharply between the resistance rates of 10% and 30% potentially highlighting a threshold for resistance for which disease control is no longer possible. Both curves plateaued from the average resistance rates of approximately 30% to 60%. The plateau of the curve of treatments mixed with captan occurred at approximately 50% disease control efficacy. In contrast, the plateau of treatments of the single-site fungicide alone occurred closer to 35%. This indicates the addition of captan provides improved disease control under scenarios of high rates of resistance when compared to treatment with the at-risk, single-site fungicide alone.

Prior to resistance emergence (0% starting inoculum)

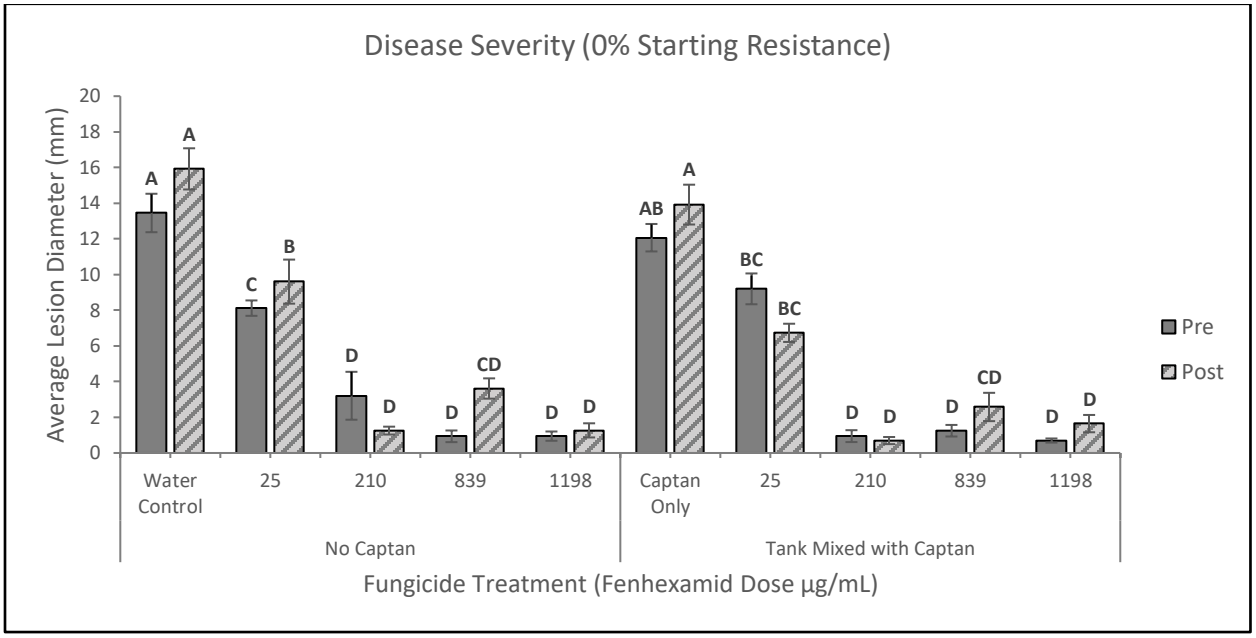

Figure 4. Disease control efficacies of various fungicide treatments on detached grape berries inoculated with only sensitive *B. cinerea* isolates. The "Pre" and "Post" data series labels designate the timing of the fungicide application as before or after artificial inoculation,

respectively. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

Under entirely sensitive scenarios, the labeled dose (1198 µg/mL) as well as two experimental lower doses of 839 µg/mL and 210 µg/mL resulted in consistently high levels of disease control. The efficacies for the same doses were not affected statistically when mixed with captan. In addition, the doses all had statistically significant differences from the water control treatment (0 µg/mL). The lowered 210 µg/mL fenhexamid dose mixed with captan when applied in a post-application manner resulted in the lowest average lesion diameter numerically. Interestingly, the lowest experimental dose (25 µg/mL) mixed with captan resulted in a moderate level of disease control often being statistically different from both the water control and labeled dose of fenhexamid. The 25 µg/mL fenhexamid dose provided significantly lower average lesion diameter values than the water control (0 µg/mL), indicating greater disease control. However, the dose also resulted in significantly higher average lesion diameter values than the higher fenhexamid doses tested, regardless of application timing or presence of captan. On one occasion the 25 µg/mL dose was statistically grouped with a higher dose. When applied in the post-infection timing with captan, the 25 µg/mL dose was statistically similar to the 839 µg/mL fenhexamid dose. The disease control efficacies resulting from the pre- and post-infection application timings are largely comparable with the only statistically significant difference observed at the 25 µg/mL dose without captan (Figure 4).

Under entirely sensitive fungal population scenarios, the application of the higher fungicide dose maintained effective disease control regardless of the presence of captan or spray timing. The higher dose resulted in greater disease control numerically in 75% of situations (12 of 18 statistically significant) when directly compared to the lower doses individually. The

higher dose was consistently more effective across application timings and the presence of a multi-site mixing partner. The mixture with a multi-site fungicide did not frequently result in statistically different disease management. When compared to the treatments of fenhexamid alone at equal doses, treatments mixed with captan resulted in lower average lesion diameters numerically in 70% of situations, however only 1 of 7 comparisons were statistically significant. The timing of the fungicide application resulted in insignificant differences in disease control when fungicide treatments of equal rates of fenhexamid and captan were compared.

Limited resistance (3% starting resistance inoculum)

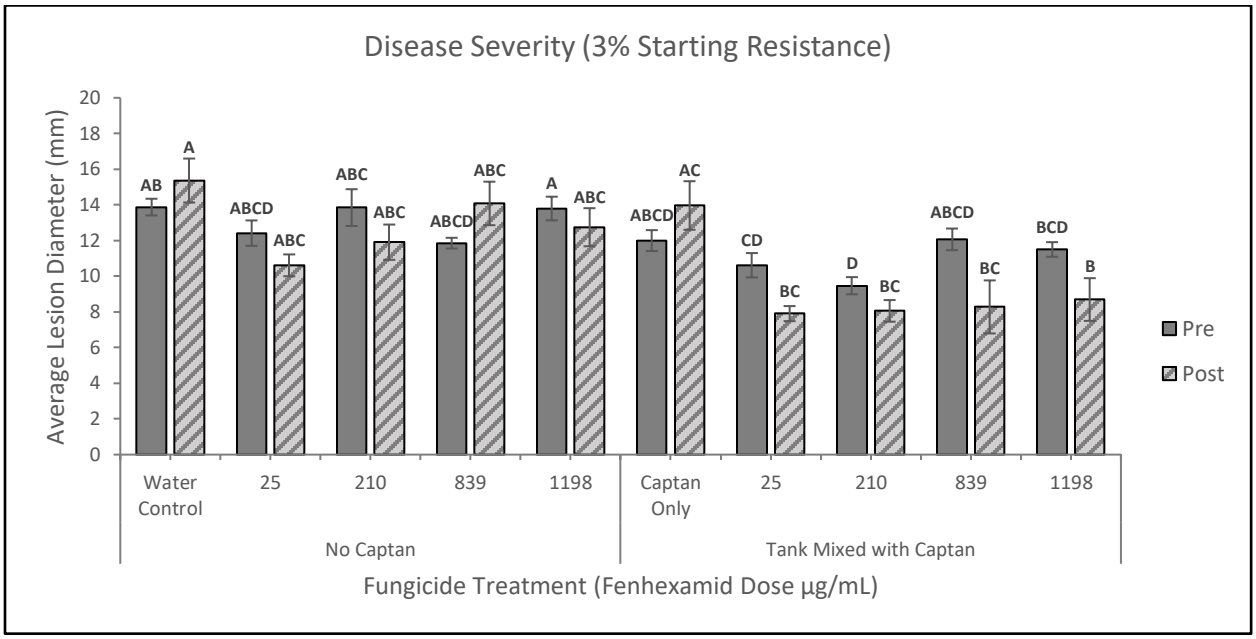

Figure 5. Disease control efficacies of various fungicide treatments on detached grape berries inoculated with both fenhexamid resistant and sensitive isolates at a concentration of 3% spores resistant to fenhexamid. The "Pre" and "Post" data series labels designate the timing of the fungicide application as before or after artificial inoculation, respectively. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

In the presence of 3% resistant populations, disease control was greatly diminished with significantly higher average lesion diameter values for all fungicide treatments compared to that of the 0% starting inoculum. Disease management was more effective in the absence of resistant

populations than that of 3% resistance ratio for all fungicide treatments (12 of 16 statistically significant) across both application timings, excluding the water and captan-only controls. Compared to the 0% resistance ratio, the average lesion diameter values averaged across all fungicide treatments increased by approximately 124% and 88% for pre- and post-infection applications, respectively. Numerically, the fungicide treatment that resulted in the lowest average lesion diameter value in the pre-infection application timing was the lowered 210 µg/mL fenhexamid dose mixed with captan. However, this treatment was not statistically different than the other doses of fenhexamid. In addition, the fungicide treatment that resulted in the lowest average lesion diameter value numerically in the post-infection application timing was the lowered 25 µg/mL fenhexamid dose mixed with captan, but no significant differences were observed when compared to the other fenhexamid doses tested. (Figure 5).

The treatments with the higher dose of the single-site fungicide resulted in lower average lesion diameter values numerically in only 25% of situations (6 out of 24) when directly compared to the lower doses. Further, none of the instances in which the higher dose resulted in lower average lesion diameter values were statistically significant. Thus, neither the higher nor lower dose seemed to be better at controlling disease in the presence of low levels of resistance to the at-risk fungicide. The mixing of a multi-site fungicide had a clear benefit on disease control in the presence of resistance. When compared to the treatments of fenhexamid alone at equal doses, treatments mixed with captan resulted in lower average lesion diameter values numerically in 90% of situations (7 of 9 statistically significant). Delayed application in a post-infection manner resulted in lower average lesion diameter values numerically in 70% of situations (3 of 7 statistically significant).

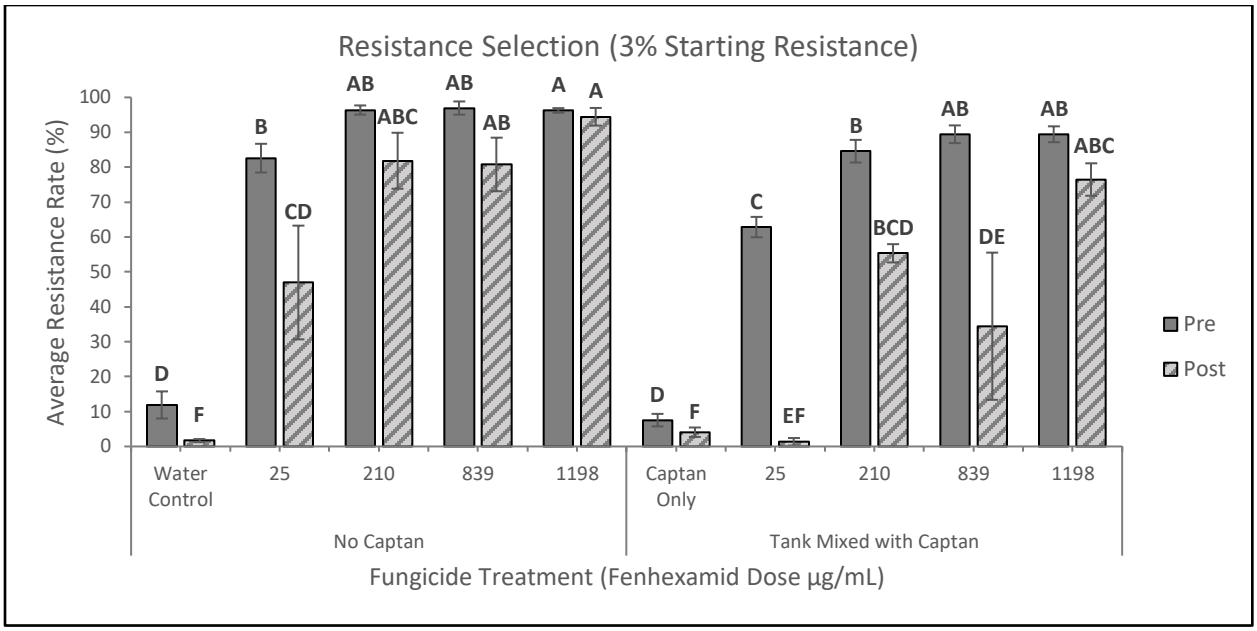

Figure 6. Resistance selection of various fungicide treatments on detached grape berries inoculated with both resistant and sensitive isolates at a concentration of 3% spores resistant to fenhexamid. The "Pre" and "Post" data series labels designate the timing of the fungicide application as before or after artificial inoculation, respectively. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

The application of fenhexamid at the labeled dose (1198 µg/mL) selected for near-complete resistance across application timings and mixing partner presence. The most effective fungicide treatment at limiting resistance selection was the 25 µg/mL fenhexamid dose, mixed with captan, and applied in the post-infection manner (Figure 6). The treatment did not significantly select for resistance as its average resistance rate was statistically grouped with the water and captan-only controls containing 0 µg/mL fenhexamid. In addition, the 25 µg/mL dose mixed with captan resulted in an average resistance rate that was statistically lower than all other doses of fenhexamid. This was consistent for both pre- and post-infection fungicide application timings.

Fenhexamid dose played a major role in the selection of resistance. The lower dose resulted in lower resistance selection numerically in 83% of the situations (11 of 20 statistically

significant). Mixture with captan had a consistent benefit of lowering average resistance rates across applications timings and fenhexamid doses. When compared to the treatment of equal fenhexamid dose alone, fungicide treatments mixed with captan resulted in lower resistance selection numerically in 90% of situations (7 of 9 statistically significant). In addition, application timing had a clear impact on resistance selection. Post-application fungicide treatments resulted in lower resistance selection numerically in 100% of situations (6 of 10 statistically significant) when comparing treatments of equal fenhexamid dose and mixture.

Intermediate resistance (10% starting resistance inoculum)

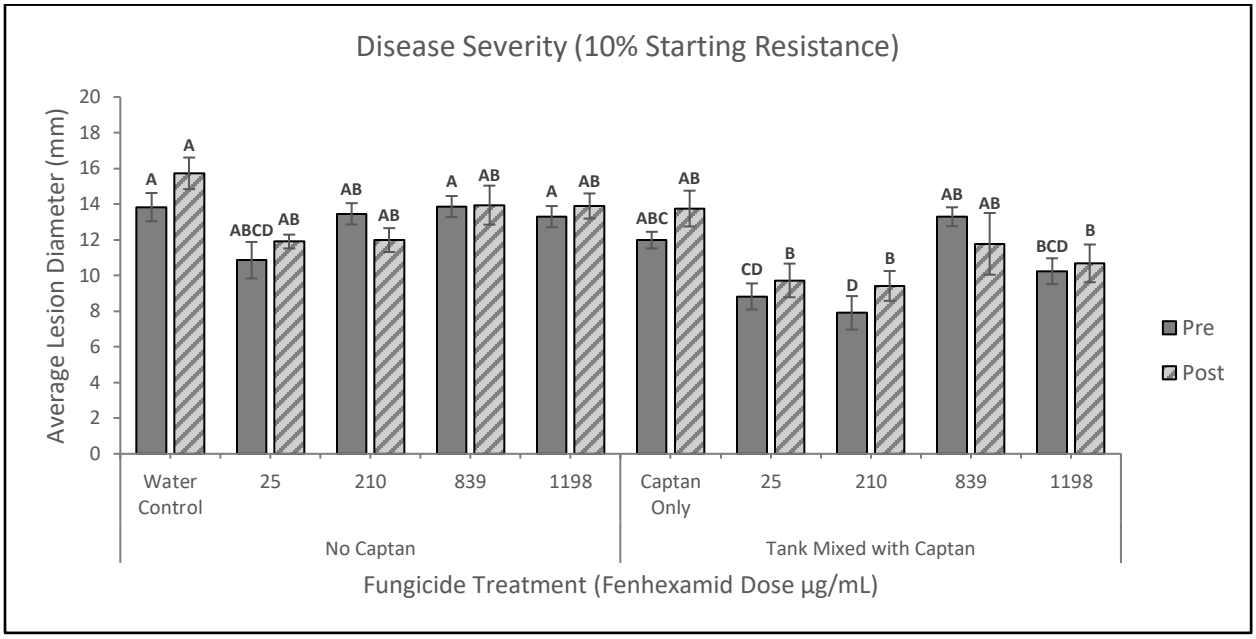

Figure 7. Disease control efficacies of various fungicide treatments on detached grape berries inoculated with both resistant and sensitive isolates at a concentration of 10% spores resistant to fenhexamid. The "Pre" and "Post" data series labels designate the timing of the fungicide application as before or after artificial inoculation, respectively. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

In the presence of an intermediately sized resistant fungal population, disease control was greatly diminished with significantly larger average lesion diameter values for all fungicide treatments compared to that of the 0% starting resistance ratio. When comparing the disease

severity in scenarios with resistance present, none of the differences between 3% and 10% fungicide treatments were significant, which may indicate that just the presence of the resistance genotype is enough to cause sufficient insensitivity to the fungicide and a widespread loss of disease control. Compared to the 3% resistance scenario, the average disease lesion diameter values averaged across all fungicide treatments increased by 2.8% and 8.2% for the pre- and post-infection application timings, respectively in the 10% starting resistance scenario. The most effective fungicide treatment for disease control numerically in both the pre-infection and post-infection application timing was the lowered 210 µg/mL fenhexamid dose mixed with captan (Figure 7). In the pre-infection timing, the treatment had statistically lower average lesion values than both the water and captan only controls (0 µg/mL) as well as all fenhexamid treatments without captan except for the 25 µg/mL dose. In the post-infection timing, the treatment resulted in statistically lower average lesion values compared to the water control (0 µg/mL) but did not result in statistical differences between any other fungicide treatments.

Treatments with the higher dose of fenhexamid were more effective in controlling disease numerically in 29% of situations (7 out of 24) when compared to the lower doses. Further, none of the instances in which the higher dose resulted in lower average lesion diameter values were statistically significant. Of the 17 situations in which the lower dose resulted in greater disease control, three were statistically significant. Thus, neither the higher nor lower dose was definitively better at controlling disease in the presence of higher levels of resistance to the at-risk fungicide. At the higher starting resistance level, 10%, mixing with a multi-site fungicide continued to provide a clear benefit to disease control. When compared to the treatments of fenhexamid alone at equal doses, treatments mixed with captan resulted in lower average lesion diameter values numerically in 100% of situations (4 of 10 statistically significant). Application

in a pre-infection manner resulted in lower average lesion diameter values numerically in 80% of situations. However, none of the differences observed were statistically significant between the application timings.

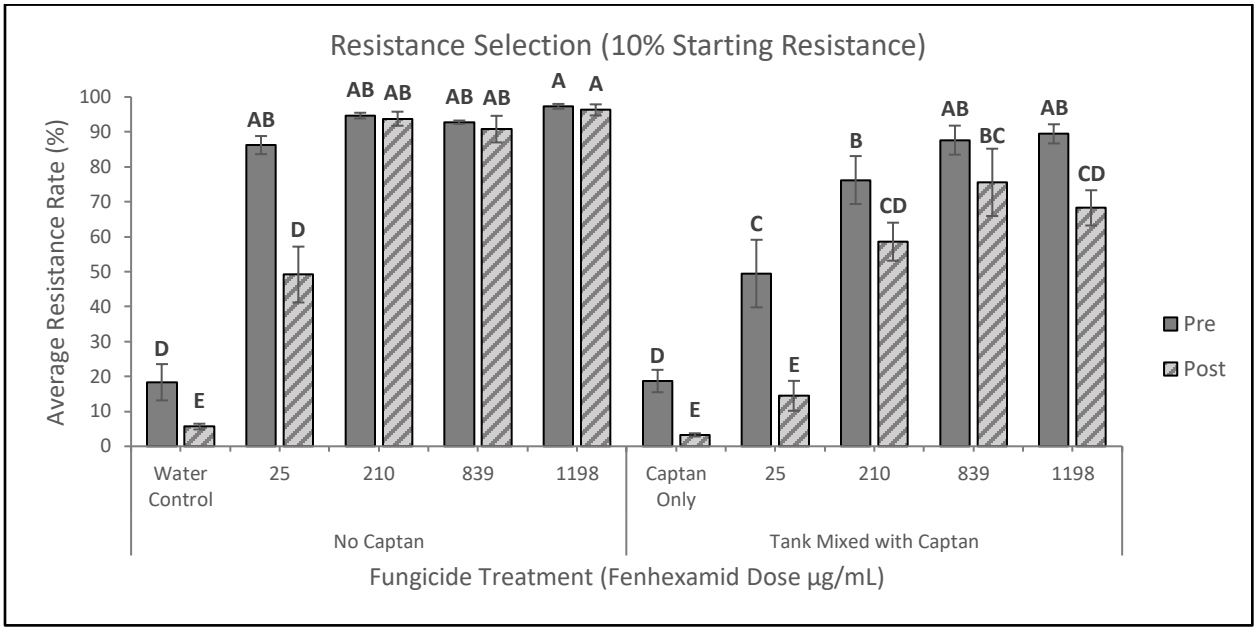

Figure 8. Resistance selection of various fungicide treatments on detached grape berries inoculated with both resistant and sensitive isolates at a concentration of 10% spores resistant to fenhexamid. The "Pre" and "Post" data series labels designate the timing of the fungicide application as before or after artificial inoculation, respectively. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

Consistent with the previous starting resistance scenario, the application of fenhexamid at the labeled dose selected for near-complete resistance across application timings and mixing partner presence. Similarly, the most effective fungicide treatment at limiting resistance selection numerically in both the pre-infection and post-infection application timings was the 25 µg/mL fenhexamid dose mixed with captan (Figure 8). In the pre-infection timing, the treatment had statistically lower average resistance rate values than all other fungicide treatments containing fenhexamid, while having statistically higher average resistance rate values than the water and captan only controls (0 µg/mL). In the post-infection timing, the treatment resulted in statistically

lower average resistance rate values compared to all other fungicide treatments containing fenhexamid, while still being statistically the same to the controls. Thus, the fungicide 25 µg/mL dose, mixed with captan, and sprayed in a post-infection manner did not significantly select for resistance to fenhexamid.

The lower dose resulted in lower resistance selection numerically in 88% of the situations (9 of 21 statistically significant). When compared to the treatment of equal fenhexamid dose alone, fungicide treatments mixed with captan resulted in lower resistance selection numerically in 90% of situations (7 of 9 statistically significant). In addition, application timing had a clear impact on resistance selection. Post-application fungicide treatments resulted in lower resistance selection numerically in 100% of situations (5 of 10 statistically significant) when comparing treatments of equal fenhexamid dose and mixture. In scenarios of 10% starting frequencies of resistance in the population, application of at a lowered dose, mixed with a multi-site fungicide, and after infection seemed to be the most efficacious strategies for resistance management. These outcomes were consistent with the trends identified from the results of the resistance selection under 3% starting inoculum.

Widespread resistance (30% starting resistance inoculum)

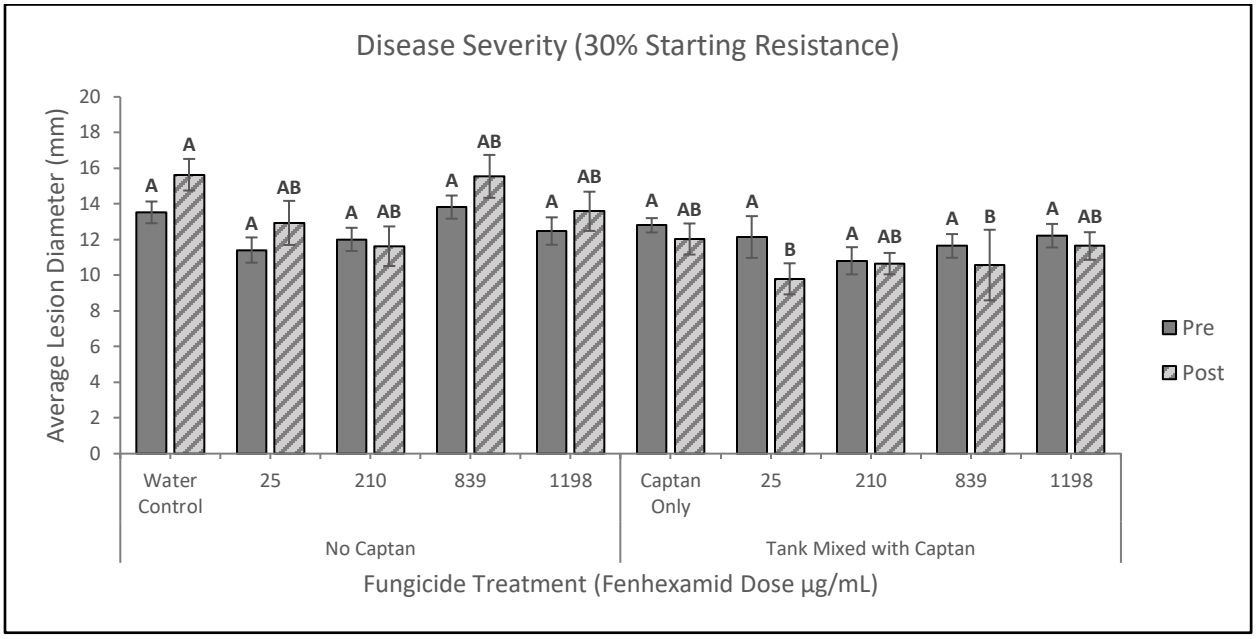

Figure 9. Disease control efficacies of various fungicide treatments on detached grape berries inoculated with both resistant and sensitive isolates at a concentration of 30% spores resistant to fenhexamid. The "Pre" and "Post" data series labels designate the timing of the fungicide application as before or after artificial inoculation, respectively. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

At the 30% resistance ratio, disease control was greatly diminished with significantly higher average lesion diameter values for all fungicide treatments compared to the 0% resistance ratio. When comparing the disease severity in scenarios with resistance present, none of the differences between 10% and 30% fungicide treatments were significant, which once again may indicate that just the presence of the resistance genotype is enough to cause sufficient insensitivity to the fungicide and a widespread loss of disease control. Compared to the 10% resistance ratio, the average disease lesion diameter values averaged across all fungicide treatments increased by 1.5% and 0.7% for pre- and post-infection applications, respectively. In the pre-infection timing, the fenhexamid dose of 210 µg/mL mixed with captan resulted in the lowest average lesion diameter numerically, however, the treatment was statistically grouped

with all other pre-infection timed fungicide treatments. Thus, at 30% starting resistance no significant differences in disease control was observed between fungicide treatments when applied preventatively. In contrast, in the post-infection timing, the lowered 25 µg/mL fenhexamid dose mixed with captan resulted in the lowest average lesion diameter numerically. However, the treatment was not statistically different from all other fungicide treatments containing fenhexamid and only retained statistical difference from the water control (0 µg/mL) (Figure 9).

The treatments with the higher dose of the single-site fungicide were more effective in controlling disease numerically in 25% of situations (6 out of 24) when directly compared to the lower doses. Further, none of the instances in which the higher dose resulted in lower average lesion diameter values were statistically significant. In the presence of widespread fenhexamid resistance, neither the higher nor lower dose controlled disease more effectively. Consistent with previous starting resistance ratios, the mixing of a multi-site fungicide continued to provide a clear benefit to disease control. When compared to the treatments of fenhexamid alone at equal doses, treatments mixed with captan resulted in lower average lesion diameter values numerically in 90% of situations (3 of 9 statistically significant). No statistically significant differences were observed between fungicide application timings.

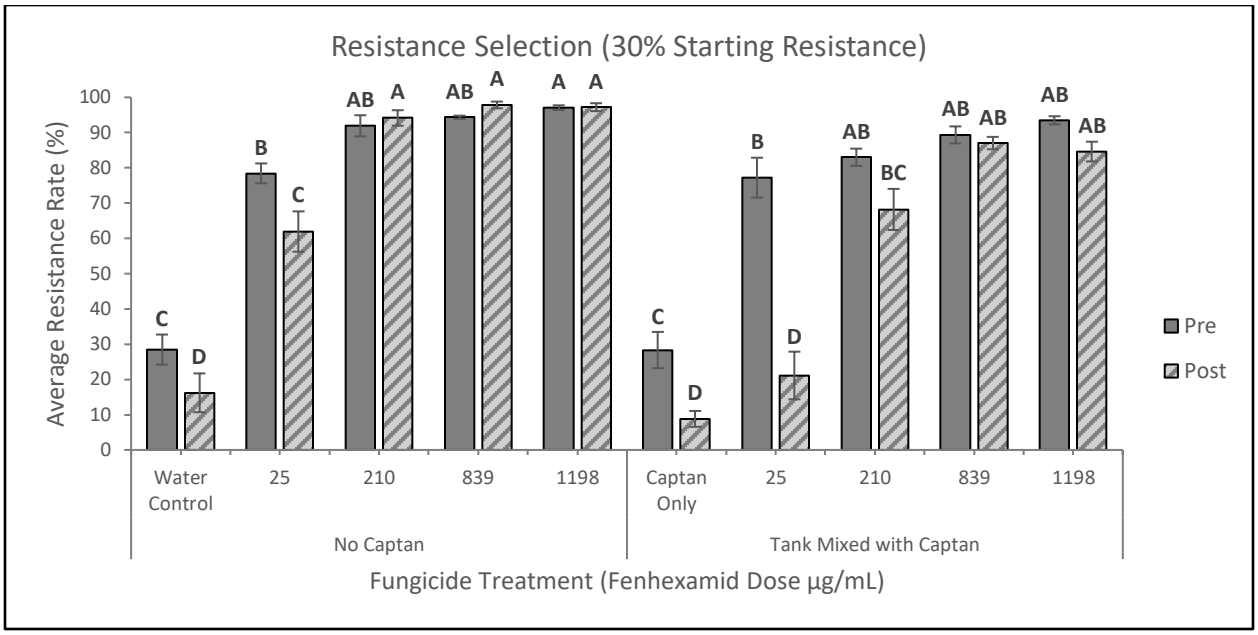

Figure 10. Resistance selection of various fungicide treatments on detached grape berries inoculated with both resistant and sensitive isolates at a concentration of 30% spores resistant to fenhexamid. The "Pre" and "Post" data series labels designate the timing of the fungicide application as before or after artificial inoculation, respectively. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

Consistent with previous starting resistance ratios, at 30% starting resistance, the application of fenhexamid at the labeled dose selected for near-complete resistance across application timings and mixing partner presence. The most effective fungicide treatment at limiting resistance selection numerically in both the pre-infection and post-infection application timings was the 25 µg/mL fenhexamid dose mixed with captan (Figure 8). However, in the pre-infection timing, the treatment was statistically the same as most other fungicide treatments containing fenhexamid, while being statistically different than the water control (0 µg/mL). In contrast, in the post-infection timing the 25 µg/mL fenhexamid dose mixed with captan treatment resulted in statistically lower average resistance rate values compared to all other fungicide treatments containing fenhexamid, while still being statistically the same as the controls. Thus,

the fungicide 25 µg/mL dose, mixed with captan, and sprayed in a post-infection manner did not significantly select for resistance to fenhexamid.

The lower dose resulted in lower average resistance rates numerically in 92% of situations (11 of 22 statistically significant). When compared to the treatment of equal fenhexamid dose alone, fungicide treatments mixed with captan resulted in lower average resistance rates numerically in 100% of situations (6 of 10 statistically significant). Post-application fungicide treatments resulted in lower resistance selection numerically in 70% of situations (5 of 7 statistically significant) when comparing treatments of equal fenhexamid dose and mixture. The benefit of a lowered fenhexamid dose, mixing with captan, and application of the fungicide after infection at managing resistance has been consistent across 3, 10, and 30% starting resistance frequencies.

Discussion

In this chapter, we determined the extent to which fungicide efficacy was lost in the presence of resistance to the at-risk fungicide, fenhexamid. In addition, we compared the effectiveness of different spray tactics including altered fungicide dose, tank mixtures, and application timings on resistance management and disease control. One of our main goals was to quantify the detrimental effect of varying rates of fungicide resistance on the ability of fungicides treatments to control their target pathogen. Disease control efficacy of the fungicide treatments declined by approximately 40% in the presence of resistance rates of 10%, regardless of the mixture with captan (Figure 3). In the case of fungicide treatments of the single-site fungicide alone, disease control efficacy was further reduced to approximately 35% in the presence of resistance rates greater than 30%. In contrast, fungicide treatments mixed with the multi-site

fungicide, captan, maintained approximately 50% disease control efficacy until resistance rates greater than 60% were reached. This is likely attributed to the nondiscriminatory nature of the captan fungicide's broad-spectrum mode of action, which retained toxicity to the *Botrytis* populations regardless of their fenhexamid resistance phenotype. These findings highlight the imperative to limit selection of resistant fungal populations in order to maintain effective disease control with extant fungicides.

Resistance management strategies are often limited in their implementation by their impact on disease control efforts. However, recommendations for fungicide applications that have the potential to effectively manage disease whilst limiting resistance selection compared to current spray recommendations should be highlighted to growers and industry stakeholders. Based on the results of the detached fruit assays in the controlled laboratory environment, under no circumstance would the labeled rate of fenhexamid be recommended for either disease control or resistance management in this pathosystem and active ingredient combination. Doses significantly lower than the labeled dose were shown to control disease similarly across all resistance scenarios, including entirely sensitive fungal populations. Consistent with the findings of previous growth chamber and modeling studies, the lower doses of the at-risk, single-site fungicide fenhexamid were shown to be more effective at limiting resistance selection in the presence of resistance when compared to the higher doses (Genet et al., 2006; van den Bosch et al., 2011; van den Bosch, Oliver, et al., 2014). The lower dose of fenhexamid resulted in lower average resistance rates numerically in 63 of 72 scenarios (31 of 63 statistically significant) (Figures 6, 8, 10). However, some previous field studies have found the lower dose to select for resistance at greater rates than higher doses of single-site fungicide (Ayer et al., 2020; Steva, 1994). Environmental factors associated with field experimentation may have contributed to the

conflicting results with Ayer et al. (2020) highlighting the necessity for further research in controlled environments. In addition, other potential factors leading to the differing results reported in Steva (1994) include the field's spray history and introduction of additional isolates with resistance from the environmental inocula that were not actively selected for by the spray programs.

Previous mathematical modeling studies have demonstrated the benefit of mixing with a multi-site fungicide for more effective disease control and resistance management (Hobbelen et al., 2013; Mikaberidze et al., 2014). However, previous work had not investigated the effect of mixing under varied frequencies of resistance. Across all four starting frequencies of resistance, fungicide treatments mixed with captan resulted in lower disease severity numerically in 35 of 40 situations (15 of 35 statistically significant) when compared to treatments of equal fenhexamid dose applied alone (Figures 4, 5, 7, 9). The curves resulting from the models of average resistance rate and disease control efficacy highlighted the benefit of mixing with captan for disease control in agreement with previous findings. Both curves showed a significant loss of disease control efficacy in the presence of fungicide resistance, however the curve of captan mixed treatments experienced a plateau at a significantly higher percentage of disease control than the curve of the single-site fungicide alone (Figure 3). In addition, mixing with captan provided statistically lower resistance selection across all three starting resistance levels. Mixing with captan resulted in lower resistance selection numerically in 28 of 30 situations (20 of 28 statistically significant) (Figures 6, 8, 10). Our results on fungicide mixtures are consistent with previous field trials reported in Forster & Staub (1996) and Oxley et al. (2008), which found mixtures to lessen shifts towards resistance in *Botrytis* and *Rhynchosporium* populations, respectively. However, conflicting results were reported in Northover (1986), which found

captan to have no impact or mitigative effect on resistance selection to the single-site fungicides benomyl or iprodione in *B. cinerea*. The differences in results between our study and that of Northover (1986) can likely be attributed to the method of resistance quantification. Northover (1986) quantified resistance through the number of isolates with resistance recovered after fungicide treatment. The drawback of this method is that it could overestimate the frequency of resistance in the fungal populations as only one resistant spore is needed to grow on the fungicide amended media for the sample to be classified as a resistant phenotype. In contrast, our study individually phenotyped fungal spores to gather a more accurate measure of the relative frequency of resistance in the population. Our methodology accounts for variability of fungal sporulation between phenotypes and thus considers potential fitness costs associated with fungicide resistance. Based on these results, the continued use of low-risk, multi-site fungicides are critical for greater disease control and lessening resistance selection for the few remaining effective single-site fungicides. Consistent with the findings of previous mathematical model studies evaluating mixing, it would be optimal to include as much low-risk, multi-site fungicide with the lowest amount of the high-risk, single-site fungicide possible while maintaining effective disease control (Elderfield et al., 2018).

Application of fungicide treatments in a curative, post-infection manner resulted in statistically similar levels of disease control throughout all resistance ratios compared to the preventative, pre-infection spray timing. Interestingly, Petit et al. (2010) found that the earlier the preventative fungicide application, the greater the protection and subsequent disease control. Instead our results are consistent with the findings of McGrath (1996) and Simpfendorfer (2016), which found that delaying fungicide application to after disease onset or after artificial inoculation was as effective in controlling disease as preventative spray programs. A possible

rationale for the differences between our findings and those in Petit et al. (2010) may be explained by the ambiguity of their fungicide applications in relation to the timing of infection. In our research, artificial inoculation allowed for precise timing of the fungicide applications either before or after infection. In contrast, the field trials conducted in Petit et al. (2010) may have had many points of *Botrytis* infection due to environmental inocula. Thus, it is difficult to ascribe a fungicide application as purely preventative or curative when the time of infection is unknown. Thus, the artificial inoculation conducted in our study as well as Simpfendorfer (2016) allow for more confident assumptions in the nature of the fungicide spray timing relative to fungal infection. These results could present a significant opportunity for growers to delay applications until fungal infection is detected in the field. Current fungal infection forecasting models rely on same-day weather data to determine the necessity of fungicide applications (Broome et al., 1995; MacKenzie & Peres, 2012). Thus, growers intervening using these models are likely making applications in a more curative, post-infection manner. In addition, the detached fruit assays showed that the post-infection application timing resulted in significantly less resistance selection across all ratios of starting resistance tested (Figures 6, 8, 10). These findings support the continued use of the disease forecasting models to determine spray applications because the delayed timing provided equivalent disease control while limiting resistance selection more effectively.

Limitations exist in the application of the detached fruit assay findings due to the nature of laboratory assays. The lack of direct UV light, consistent temperature, and absence of moisture to remove fungicide residue, all of which may degrade the fungicide in greenhouse or field settings, make laboratory conditions the most ideal setting for the efficacy of the fungicides being tested (Wołejko et al., 2016). As the lowering of the at-risk, single-site fungicide dose was

a central conclusion drawn from the assays it is imperative to further validate the results. Although significantly lowered doses of fenhexamid were able to control disease comparably to the labeled dose, environmental factors present in the greenhouse or field settings may alter and impact the efficacy of the lowered doses and thus overall recommendations on dosage. Despite this, the fundamental trends for best managing resistance are clear for this pathosystem and active ingredient combination. Recommendations for the dose of the at-risk, single-site fungicide should focus on the lowest possible rate for maintenance of effective disease control. Unnecessary application at the labeled dose may be incurring greater resistance selection, higher input costs for growers, and higher exposure for applicators all while providing little to no benefit on disease control. Mixing with a multi-site fungicide often resulted in significantly better disease control and lower resistance selection making it an important tactic for deployment against *Botrytis* populations with varied frequencies of resistance. In addition, fungicide application in the post-infection manner was as effective as preventative sprays at controlling disease. Importantly, post-infection fungicide applications were determined to result in lower resistance selection than preventative, pre-infection, fungicide applications. Under controlled laboratory conditions, the application of the single-site fungicide at a lowered rate, mixed with captan, and sprayed in a post-infection manner are the most effective methods for limiting resistance selection regardless of the frequency of resistance already present in the fungal population. Further, these recommendations would be expected to largely result in equivalent levels of disease control compared to preventative applications at the labeled dose of the single-site fungicide based on our findings.

Chapter 2: Validation of fungicide spray tactics for managing disease and limiting resistance selection in *Botrytis cinerea* through greenhouse assays

Introduction

Botrytis bunch rot (BBR) is a common wine grape disease in the Mid-Atlantic region resulting in significant economic losses. *Botrytis cinerea* infection of grape clusters causes the proliferation of gray conidial masses and overwhelming rot of the fruit (Wilcox et al., 2015). Environmental and climatic conditions conducive to *Botrytis* infection and colonization are cool temperatures and high moisture (Bettiga, 2013). Infection of highly susceptible anthers at bloom can result in latent *Botrytis* infections (Nair et al., 1995). These latent infections often result in heightened disease incidence at later physiological stages of berry maturity, particularly after veraison (Keller et al., 2003). Further, secondary infections often occur within the same cluster on adjacent berries or from the aerial spread of spores to nearby clusters (González-Domínguez et al., 2015). As a primary pathogen on grape clusters, *B. cinerea* can gain entry and cause infection in both wounded and unwounded berries. Common cultural control methods for the disease are to limit mechanical wounding on the berries, removal of infected plant material to limit primary infections from overwintering structures, and canopy thinning (Bettiga, 2013). Despite these efforts, chemical applications remain the most effective control methods of the disease throughout the growing season (Wilcox et al., 2015). Targeted fungicide applications during weather conditions considered to be high risk for *Botrytis* infection remain critical for disease control (Bettiga, 2013).

BBR is commonly controlled through the application of fungicides at four important physiological stages of grape cluster development. Current spray guidance recommends applications at mid-bloom to prevent latent infections, prior to berry touch, early veraison to protect newly susceptible berries, and a few weeks after veraison to protect against secondary spread before harvest (Wilcox et al., 2015). Fungicides commonly used for control of BBR include phenylpyrroles, succinate dehydrogenase inhibitors, anilinopyrimidines, dicarboximides, and hydroxyanilides all of which possess single-site modes of action (Alzohairy et al., 2021; Leroux et al., 2002). These chemical groups are typically rotated or used in a mixture with a multi-site fungicide such as captan for BBR control throughout the growing season. Fenhexamid, a hydroxyanilide, has high efficacy and is relatively inexpensive making it an excellent fungicide in spray programs. However, resistance development has become a significant threat to the continued use of fenhexamid for the control of BBR. Fenhexamid resistance has been detected and quantified across the continental United States. In California vineyards, resistant isolates were collected at a frequency of 13.7% (Saito et al., 2019). Across four years, fenhexamid resistance rates increased in Michigan vineyards from 3.4% to 38.4% (Alzohairy et al., 2021). Although common for grapevine disease management, fenhexamid is often sprayed for disease control in other small fruit crops. Resistance frequencies of 17% and 30% have been observed in Carolina and Florida strawberry fields, respectively (Amiri & Peres, 2014; Grabke et al., 2013). In addition, resistance to fenhexamid was observed at 29.3% in California and Washington blueberry fields (Saito et al., 2016).

Previous laboratory detached fruit assays described in the chapter 1 have highlighted key prospective fungicide application strategies for controlling disease and limiting resistance selection across a diverse range of frequencies of fungicide resistance. Despite this, clear

limitations exist in the application of these findings. Laboratory assays have limited exposure to ultraviolet light (UV), moisture events, and varying temperatures. These external factors can have a significant impact on the efficacy of the fungicides applied. Although a nominal dose of fungicide may be formulated in the lab, the effective dose once sprayed in the greenhouse or field may be lower due to imperfect spray coverage or dilution from excess moisture. In addition, the degradation of the active ingredient caused by heat and UV exposure may limit the efficacy of the fungicides applied. Thus, the primary objective is to validate a previously identified lower dose of fenhexamid and involvement of captan for BBR control and fungicide resistance management using potted grapevines in the greenhouse.

Materials and methods

Inoculation and fungicide applications

In the summer of 2021 and 2022, potted grapevines, *Vitis vinifera* 'Cabernet Sauvignon', on drip irrigation were grown at the greenhouse research complex of University of Maryland College Park to provide clusters for in vivo testing of the factors examined in the detached fruit assays. At approximately 50% bloom, artificial inoculation with a mixture of eight *Botrytis cinerea* isolates was conducted. Spore suspensions containing 0, 5, and 15 percent fenhexamid-resistant spores were sprayed onto their respectively labeled blooms. The varied rates of fenhexamid-resistant spore suspensions were made by adding four *Botrytis cinerea* isolates previously collected and identified as fenhexamid-resistant (Cosseboom & Hu, 2021; Hu et al., 2018). Plastic bags were used to cover the blooms immediately following the spray inoculation to retain moisture and facilitate conditions conducive for fungal infection. Two days after inoculation the bags were removed and the first, bloom stage, fungicide application was

conducted. Three subsequent fungicide applications were conducted at the major cluster physiological stages of bunch closure, veraison, and pre-harvest, as normally recommended for BBR management. Six distinct fungicide treatments were tested. Three of these treatments included only the fenhexamid dose from the fungicide label of 1198 µg/mL, an exploratory lower dose of 210 µg/mL, or water control. The other three fungicide treatments used the fenhexamid doses listed above in a mixture with captan at medium dose of 4793 µg/mL as outlined in (Table 3). Fungicide applications were conducted using handheld spray bottles to provide better, more consistent coverage on the grape clusters (replicates). The 2021 greenhouse experiment included a total of 144 replicates and the 2022 greenhouse experiment included a total of 432 replicates. All treatments were randomized throughout the greenhouse cell, but only replicates requiring the same fungicide treatment were included on the same grapevine to minimize spray drift between clusters on the same grapevines that would confound treatments.

Table 3. Outline of the active ingredient concentrations of the six fungicide treatments in the greenhouse experiments.

Fungicide Treatment	Fenhexamid[x] Concentration (µg/mL)[z]	Captan[y] Concentration (µg/mL)[z]
1	1198	0
2	210	0
3	0	0
4	1198	4793
5	210	4793
6	0	4793

[x] Trade name Elevate 50 WDG
[y] Trade name Captan 80 WDG
[z] Fungicide doses were determined based on the application rate of 50 gallons of water per acre.

Greenhouse sampling

After each fungicide application, three berries were harvested from each grape cluster replicate. The harvested berries were cut hemispherically and plated onto potato dextrose agar

(PDA). All plates were incubated at 22°C in alternating light and dark conditions. The plates were monitored for *B. cinerea* growth for approximately 7 days, and all resulting isolates were sub-cultured. The *Botrytis* infection rate for each inoculum and fungicide treatment combination was recorded. In addition to the sampling method conducted for all four physiological time points, a final analysis of the clusters was conducted after the last, pre-harvest fungicide application. Specifically, the entire clusters were harvested, and the individual berries were removed with the peduncles still attached to not create wounds. The berries were incubated at 22°C in humid conditions to promote fungal vegetative growth. The berries were monitored for BBR symptoms, and the disease incidence was recorded.

Fenhexamid resistance determination

Once all isolates were recovered, they were placed under light conditions to induce sufficient sporulation such that spore suspensions could be obtained. Autoclaved tap water (250μL) was used to flood a section of each plate, disposable L-shaped spreaders were used to dislodge the spores, and the water was withdrawn using a pipette to be stored in a 1.5mL Eppendorf tube. The resulting spore suspensions were vortexed to homogenize the suspension and plated onto malt extract agar (MEA) plates containing the discriminatory dose of 50 ppm fenhexamid (Weber & Hahn, 2011). The MEA plates with the spore suspensions were incubated at 22°C in entirely dark conditions for 18 hours. Then the germination tubes of the spores were visualized under a microscope to determine their resistance phenotype. Ten spores were phenotyped at three random locations for a total of 30 spores for each plate.

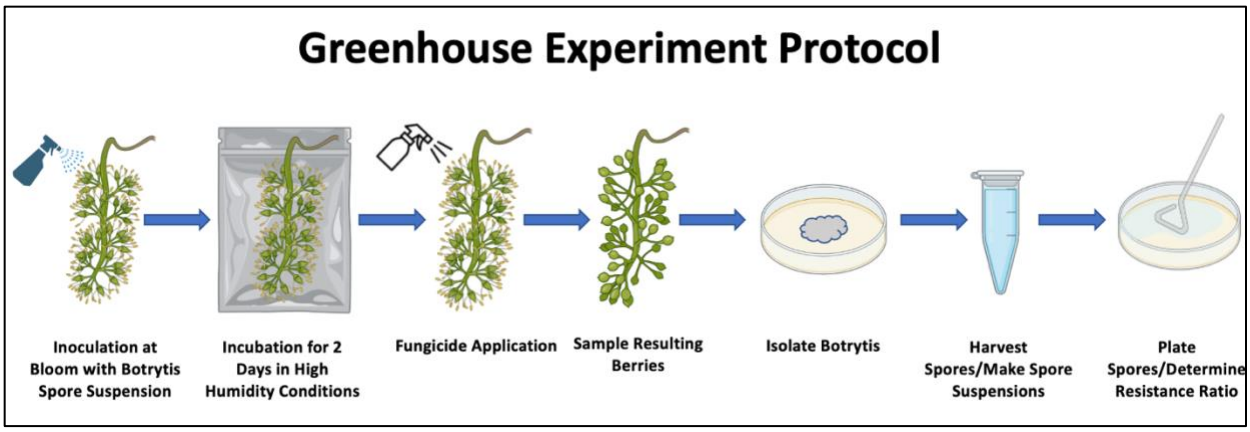

Figure 11. Protocol for the greenhouse experiments on whole grape clusters. Artificial inoculation occurred at bloom with *B. cinerea* isolates at varying ratios of resistance. After incubation, fungicide applications were applied at physiologically relevant timepoints. After each timepoint and application, individual berries were sampled, *Botrytis* was isolated, and the resistance ratio for each sample determined.

Statistical analysis

Given that the datasets resulting from the 2021 and 2022 seasons did not follow a normal distribution, the infection rate and fenhexamid resistance rate datasets were transformed into ranked averages and combined using the JMP Pro 15 software. Then the non-parametric ranked averages were used to compare the fungicide treatments against one another under the varied starting resistance scenarios. Using the Kruskal-Wallis test, statistically significant differences between fungicide treatments were identified for both the infection rate and fenhexamid resistance rate datasets across each of the four physiological time points sampled. Further, the whole cluster infection rate data was analyzed using the same methods. To determine the effect of individual factors on both disease severity and resistance selection, fungicide treatments varying only in the factor of interest were compared. The results of all comparisons of a particular factor were compiled despite differences in the other factors that were tested simultaneously. Thus, herein the term “situations” is used to reflect the diversity of other factors

that were blocked during the comparison of the factor of interest. As a result, the term may carry a unique meaning dependent on the individual factor of interest being discussed.

Results

Prior to resistance emergence (0% starting inoculum)

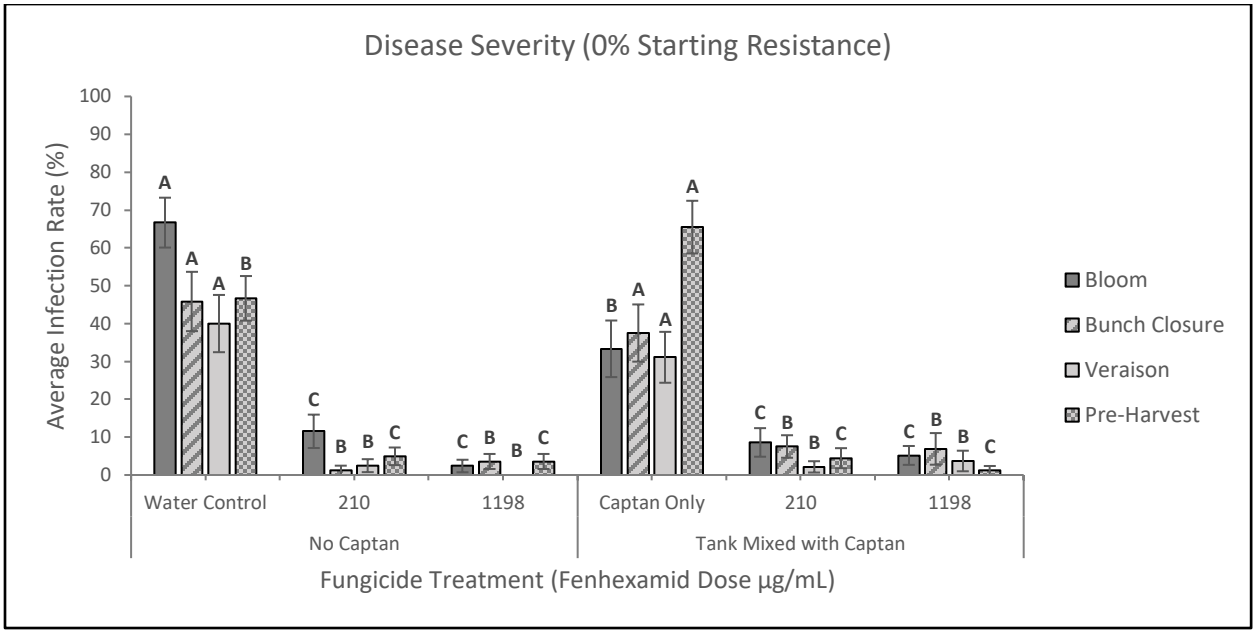

Figure 12. Disease control efficacies of various fungicide treatments on grape clusters inoculated with sensitive *B. cinerea* isolates. The data included resulted from spray inoculation at bloom. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

In the absence of fenhexamid-resistance, the efficacy of all fungicide treatments was high and statistically better than the water-only (0 µg/mL) treatment throughout all physiological time points except for the captan-only treatment. In addition, all fungicide treatments containing fenhexamid were statistically grouped with one another indicating the maintenance of similar disease control throughout all physiological stages, regardless of fenhexamid dose and presence of a multi-site mixing partner (Figure 12). Disease control was similar across all physiological time points including pre-harvest.

Under entirely sensitive fungal population scenarios, the application of the higher fungicide dose resulted in greater disease control numerically in 75% of situations (6 of 8) when directly compared to the lower dose. However, none of the differences were statistically significant and thus the experimentally lowered dose of 210 µg/mL fenhexamid was as effective as the labeled dose at managing the disease. Further, when compared to the treatments of fenhexamid alone at equal doses, the mixture with a multi-site fungicide resulted in lowered infection rates numerically in only 58% of situations (7 of 12) across the four physiological time points. Only one of the comparisons resulted in statistically significant differences potentially indicating that the mixing with captan is unnecessary in the absence of resistance to the at-risk single-site fungicide.

Limited resistance (5% starting inoculum)

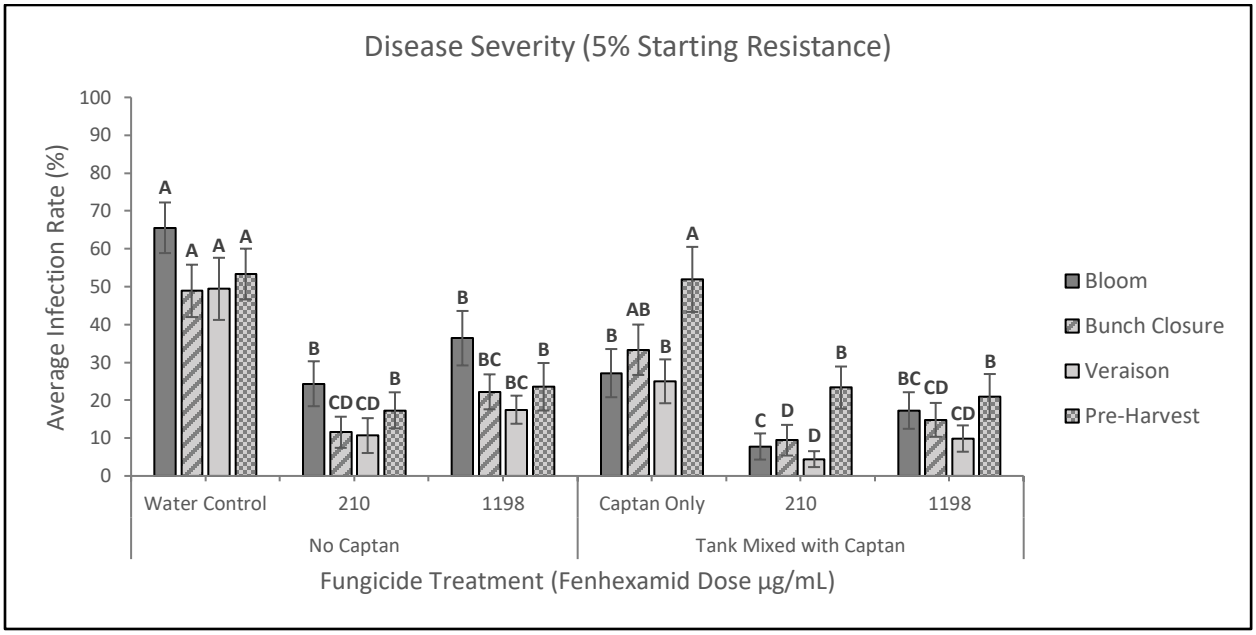

Figure 13. Disease control efficacy of various fungicide treatments on grape clusters inoculated with resistant and sensitive *B. cinerea* isolates at a concentration of 5% spores resistant to fenhexamid. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

At the 5% resistance ratio, the efficacy of the fungicide treatments for controlling disease was decreased compared to the 0% resistance ratio across physiological time points. All the fungicide treatments containing fenhexamid resulted in statistically lower infection rates than both the water-only (0 µg/mL) and captan-only (0 µg/mL mixed with captan) control treatments throughout all physiological time points, except for one treatment at bloom (Figure 13). When comparing the four fungicide treatments containing fenhexamid, the lowered dose (210 µg/mL) mixed with captan provided statistically lower disease infection rates at bloom than the two treatments of fenhexamid without captan. However, the labeled dose (1198 µg/mL) mixed with captan resulted in statistically similar infection rates at bloom to the lowered dose mixed with captan. At bunch closure and veraison, all four fungicide treatments containing fenhexamid were statistically grouped together, except for the lowered fenhexamid dose mixed with captan which had statistically lower infection rates than the labeled fenhexamid dose without captan. Finally, at pre-harvest, all four fungicide treatments were statistically grouped together and resulted in similar disease control. Consistent with the entirely sensitive (0%) resistance ratio, infection rates were similar across all physiological time points including pre-harvest.

The application of the higher fungicide dose resulted in greater disease control numerically in only 12% of situations (1 of 8) when directly compared to the lower dose. Further, none of the differences were statistically significant. Thus, the experimentally lowered dose of 210 µg/mL fenhexamid was as effective as the labeled dose at managing the disease. Further, when compared to the treatments of fenhexamid alone at equal doses, the mixture with a multi-site fungicide resulted in lowered infection rates numerically in 100% of situations (12 of 12) across the four physiological time points. One of the comparisons resulted in statistically

significant differences, however, a trend exists advocating for the usage of a multi-site mixing partner in the presence of low levels of resistance.

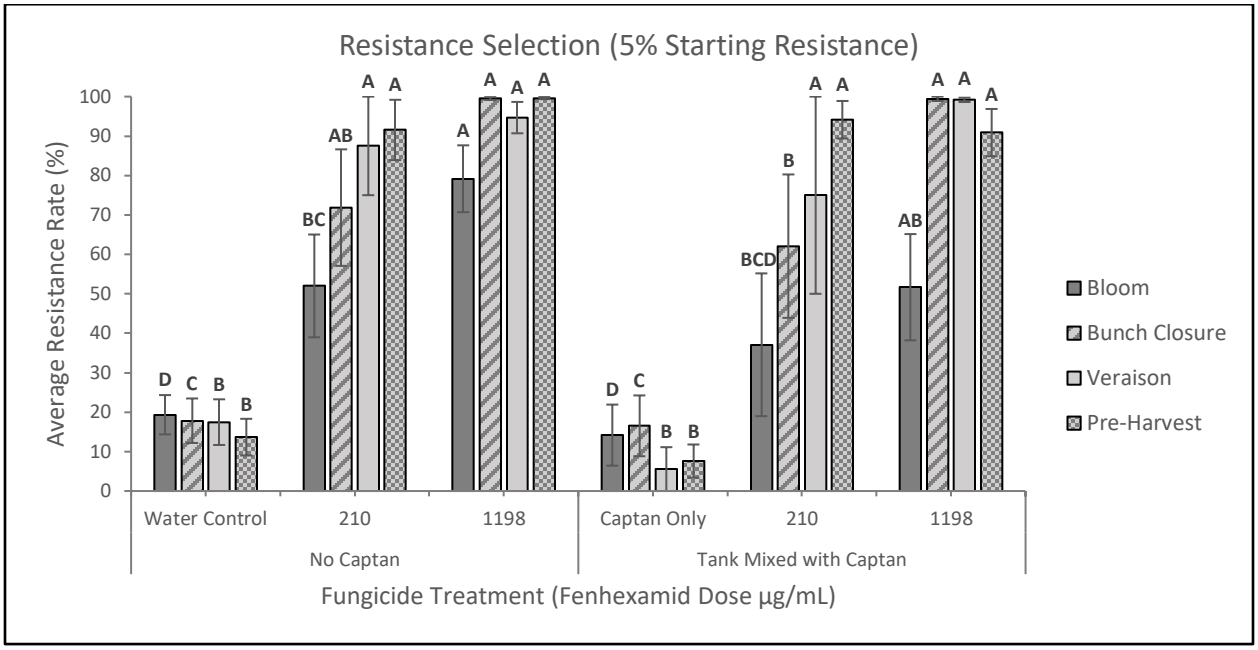

Figure 14. Resistance selection of various fungicide treatments on grape clusters inoculated with resistant and sensitive *B. cinerea* isolates at a concentration of 5% spores resistant to fenhexamid. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

The largest increases in resistance rates were observed at the first physiological time point from the initial resistance ratio. Fungicide applications at the following time points resulted in further, yet smaller increases in average resistance rates. All the fungicide treatments containing fenhexamid resulted in statistically higher average resistance rates than both the water-only (0 µg/mL) and captan-only (0 µg/mL mixed with captan) control treatments throughout all physiological time points, except for one treatment at bloom (Figure 14). The lower fenhexamid dose (210 µg/mL) mixed with captan resulted in resistance rates that were statistically grouped with both the control treatments at bloom, indicating the lower dose mixed with captan most successfully managed resistance after a single fungicide application. At bunch closure, the lower dose mixed with captan resulted in statistically lower resistance rates than the

labeled dose regardless of the captan mixture. However, the lower dose mixed with captan was no longer grouped with the controls indicating an increased level of resistance selection after the second fungicide application. At veraison and pre-harvest, all four fungicide treatments were statistically grouped together and resulted in similar resistance selection.

The application of the lower fungicide dose resulted in lower resistance rates numerically in 88% of situations (7 of 8) when directly compared to the labeled dose. In addition, two of the situations had statistically significant differences. The experimentally lowered dose, 210 µg/mL, was more effective than the labeled dose at limiting resistance selection to the single-site fungicide. Further, when compared to the treatments of fenhexamid alone at equal doses, the mixture with a multi-site fungicide resulted in lower rates of resistance numerically in 83% of situations (10 of 12) across the four physiological time points. None of the comparisons resulted in statistically significant differences, however, a trend supporting the usage of a multi-site mixing partner for lessening resistance selection exists.

Intermediate resistance (15% starting inoculum)

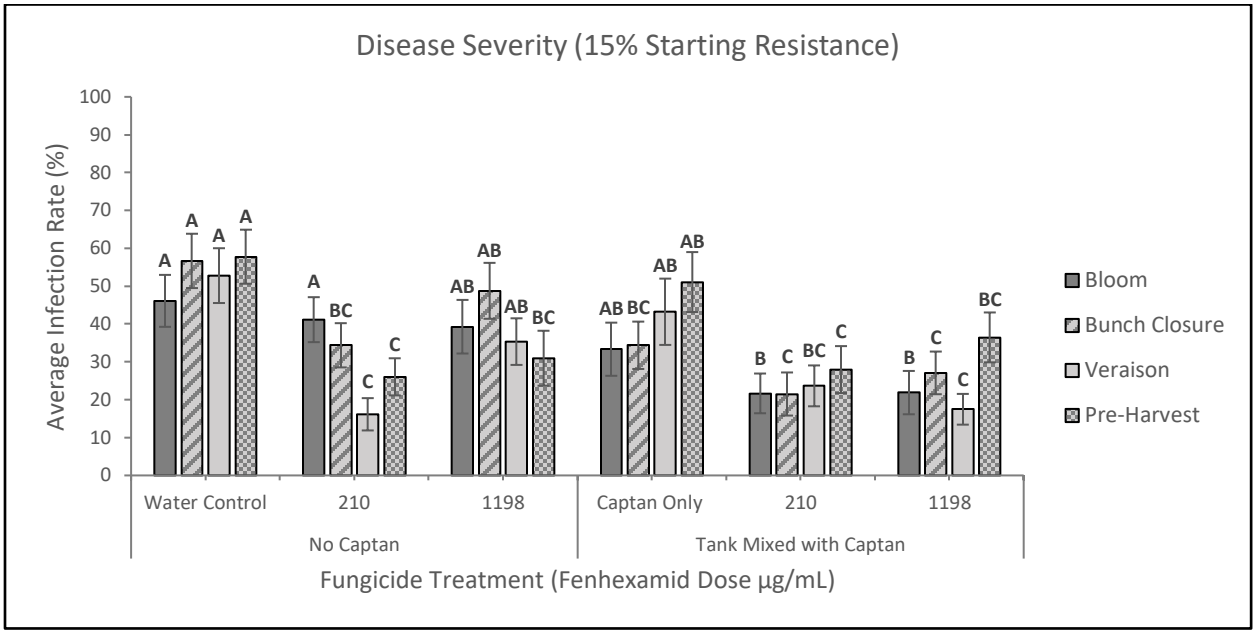

Figure 15. Disease control efficacies of various fungicide treatments on grape clusters inoculated with resistant and sensitive *B. cinerea* isolates at a concentration of 15% spores resistant to fenhexamid. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

At the higher, 15%, starting resistance ratio, the efficacy of the fungicide treatments for controlling disease was decreased compared to the 0% resistance ratio across all physiological time points. In addition, some fungicide treatments no longer maintained statistical differences from the water-only (0 µg/mL) and captan-only (0 µg/mL mixed with captan) control treatments throughout all physiological time points further indicating a widespread loss of efficacy (Figure 15). When comparing the four fungicide treatments containing fenhexamid, the two treatments mixed with captan provided statistically lower infection rates at bloom than the water control. However, the same doses of fenhexamid without captan did not have significantly lower levels of infection than the water control at bloom highlighting a clear benefit of mixing with captan at higher rates of resistance. At bunch closure, all treatments containing fenhexamid were statistically grouped together with lower infection rates than the water control, except for the

labeled dose (1198 µg/mL) of fenhexamid alone which continued to provide worsened disease control. Finally, at pre-harvest, all four fungicide treatments were statistically grouped together and resulted in similar disease control. Consistent with both the entirely sensitive (0%) and early emerged resistance (5%) resistance ratios, the infection rate remained relatively similar across all physiological time points for each fungicide treatment.

The application of the higher fungicide dose resulted in greater disease control numerically in only 38% of situations (3 of 8) when directly compared to the lower dose. Further, none of the differences were statistically significant indicating that the lowered dose, 210 µg/mL, was as effective as the labeled dose for disease management. When compared to the treatments of fenhexamid alone at equal doses, the mixture with a multi-site fungicide resulted in lowered infection rates numerically in 83% of situations (10 of 12) across the four physiological time points. Only two of the comparisons resulted in statistically significant differences, however, once again a trend exists advocating for the usage of a multi-site mixing partner in the presence of higher levels of resistance.

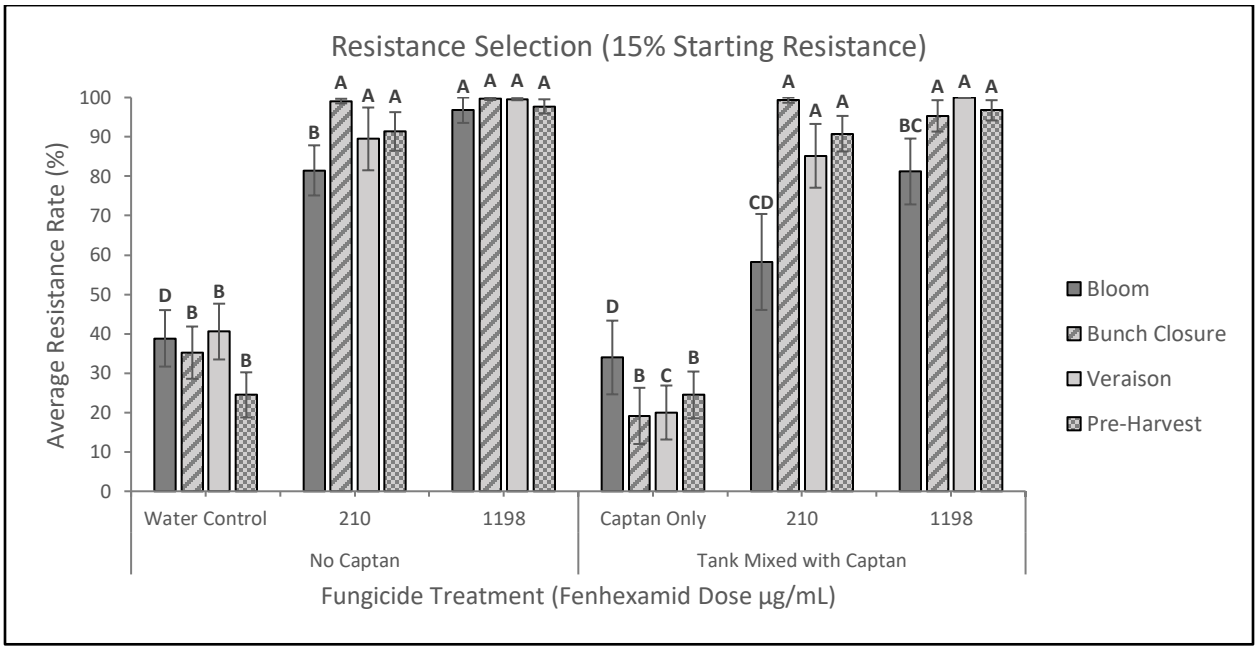

Figure 16. Resistance selection of various fungicide treatments on grape clusters inoculated with resistant and sensitive *B. cinerea* isolates at a concentration of 15% spores resistant to fenhexamid. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

All the fungicide treatments containing fenhexamid resulted in statistically higher average resistance rates than both the water-only (0 µg/mL) and captan-only (0 µg/mL mixed with captan) control treatments throughout all physiological time points, except for one treatment at bloom (Figure 16). The lower fenhexamid dose, 210 µg/mL, mixed with captan resulted in average resistance rates that were statistically grouped with both control treatments at bloom, indicating that it was the most effective treatment at managing resistance and limiting resistance selection. In addition, the treatment was statistically different from both fungicide treatments of fenhexamid without captan highlighting the benefit of using a lowered dose of the at-risk fungicide and mixing parting for more effective resistance management. At the bunch closure, veraison, and pre-harvest physiological time points all four fungicide treatments containing fenhexamid were statistically grouped together and resulted in similar resistance selection. At the higher 15% starting resistance ratio, more than one consecutive fungicide application containing fenhexamid selected for near complete fenhexamid-resistance within the fungal population regardless of dose or the presence of a multi-site mixing partner.

The application of the lower fenhexamid dose resulted in lower resistance rates numerically in 88% of situations (7 of 8) when directly compared to the higher labeled dose with one of the situations being statistically significant. Similar to the 5% starting resistance ratio, the experimentally lowered dose, 210ppm, was more effective than the labeled dose at limiting resistance selection to the single-site fungicide. Further, when compared to the treatments of fenhexamid alone at equal doses, the mixture with a multi-site fungicide resulted in lower rates

of resistance numerically in 83% of situations (10 of 12) across the four physiological time points. Three of the comparisons resulted in statistically significant differences, supporting use of a multi-site mixing partner for resistance management to the at-risk single-site fungicide.

Whole Cluster Infection Analysis

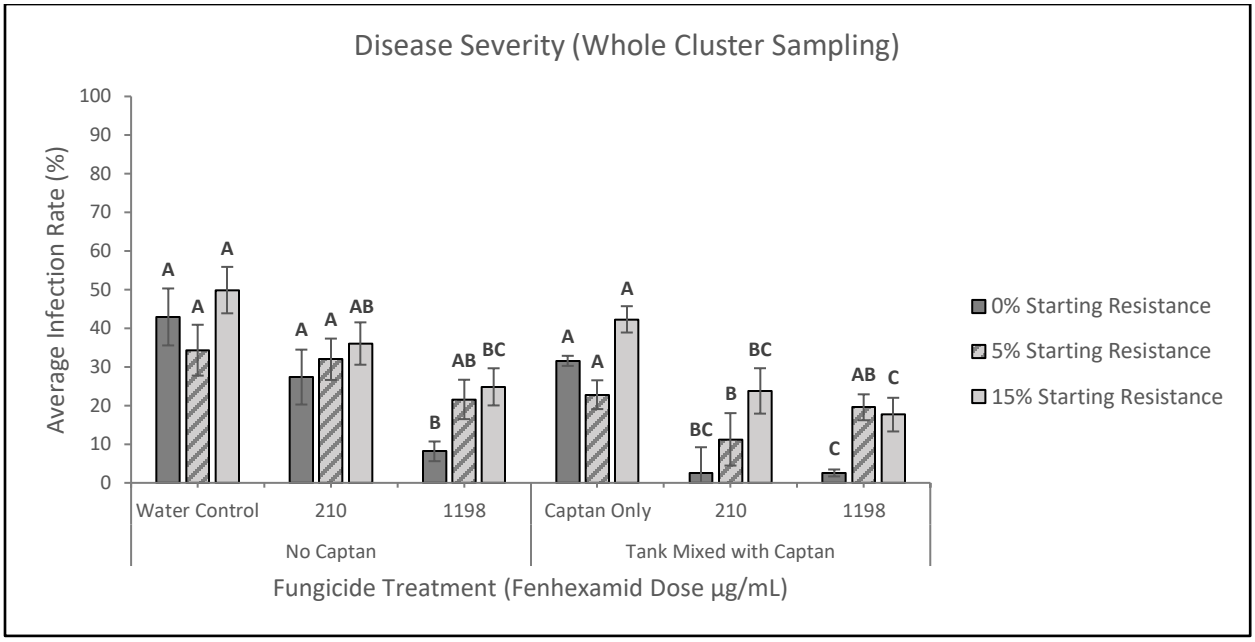

Figure 17. Post-harvest disease control efficacies of various fungicide treatments on grape clusters inoculated with *B. cinerea*. Whole grape clusters were harvested as a more comprehensive method to assess the rate of infection and better quantify disease severity at the end of the growing season. Fungicide treatments that were tank mixed with captan contained an active ingredient concentration of 4793 µg/mL.

In addition to the sampling conducted after the pre-harvest fungicide application, the entire clusters were harvested for all replicates. The whole clusters were incubated in the laboratory setting to quantify the level of *Botrytis* infection more accurately at the end of the growing season. Thus, the data included in (Figure 17) is the result of four consecutive fungicide applications. The presence of captan was often responsible for statistical differences between the four fungicide treatments containing fenhexamid. Under entirely sensitive scenarios, the most

effective fungicide treatment for controlling disease was the labeled dose (1198 µg/mL) mixed with captan, which had significantly lower average infection rates than both fenhexamid treatments that were not mixed with captan. At the 5% starting resistance ratio, the most effective treatment for disease control was the lower, 210 µg/mL, fenhexamid dose mixed with captan. The treatment resulted in statistically lower infection rate averages than the equal fenhexamid dose without captan. Finally, the higher 15% starting fenhexamid-resistant fungal population was most effectively controlled by the labeled dose of fenhexamid (1198 µg/mL) mixed with captan. However, similar to both the 0% and 5% starting resistance ratios, the lowered dose mixed with captan had statistically similar results to the 1198 µg/mL fenhexamid dose. Importantly, the 210 µg/mL and 1198 µg/mL fenhexamid treatments containing captan were not statistically different from one another at any inoculum starting resistance rate.

Discussion

In this chapter, we attempted to validate the findings and trends identified from our previous laboratory detached fruit assays. Specifically, we compared the effectiveness of different spray tactics including altered fungicide dose and tank mixtures on resistance management and disease control. These factors were tested on whole grape clusters under varied frequencies of fenhexamid resistance in controlled greenhouse environmental conditions. Throughout the greenhouse assays, the rate of fenhexamid resistance at each physiological time point and subsequent fungicide application was quantified. Importantly, the most significant increase in resistance frequencies was after the first fungicide application, potentially indicating that a single inappropriate application of the fungicide may negatively impact resistance management efforts more severely than previously understood. In contrast, the rate of infection

across physiological time points remained relatively steady. This was an expected result given that artificial inoculation was conducted only once at bloom. Thus, all points of sampling throughout the growing season measured the rate of latent infections in the replicates. As a result, largely the same level of BBR was observed post-harvest.

Despite significant results from the detached fruit assays demonstrating that the lower dose of fenhexamid could provide effective disease control while selecting for resistance less than the labeled dose, questions remained as to whether this dynamic would be consistently expressed in the greenhouse setting. The laboratory environment in which the lower dose was previously tested as part of the detached fruit assays was optimal conditions for the fungicide. In contrast, the environmental factors present in the greenhouse were posited to make the experimentally lowered fenhexamid dose impractical for effective disease control. In addition, Ayer et al. (2020), a field experiment with conclusions in conflict with our findings on fenhexamid dose, explicitly stated that future research in controlled environments was necessary. In our greenhouse assays, in the absence of fenhexamid-resistant isolates, the labeled dose (1198 µg/mL) was more effective at managing disease and resulted in lower average infection rates numerically in 75% of situations (6 out of 8) compared to the lower dose (210 µg/mL). In contrast, when the fenhexamid-resistance genotype was present, the lower dose (210 µg/mL) was more effective at managing disease and resulted in lower average infection rates numerically in 75% of situations (12 out of 16) compared to the labeled dose. Importantly, none of the situations in which the labeled dose resulted in better disease management were statistically significant. Thus, the significantly decreased dose of 210 µg/mL was as effective at managing the level of *Botrytis* infection as the labeled dose (1198 µg/mL) across varied resistance scenarios and all physiological time points. Supporting the use of the lower dose of fenhexamid further, the

decrease in dose of the at-risk fungicide selected for resistance less. Based on the data from the 5% and 15% starting resistance frequencies, the lower fenhexamid dose resulted in lower average resistance rates numerically in 88% of situations (14 out of 16) when compared to the higher, recommended dose on the fungicide label. Only three of these situations were statistically significant, but in combination with the detached fruit assay results, the conclusions are clear. A previous study testing the effect of lower single-site fungicide dose on resistance selection on grapevines reported findings consistent with the detached fruit assays and greenhouse trial results. The trial in Genet et al. (2006) tested varied fungicide doses on whole chardonnay plants in growth chambers. These conditions are largely analogous to our experimental design and strengthen our findings and the prospect of lowered single-site dose usage. The labeled dose of the single-site fungicide fenhexamid is too high for greenhouse applications. Application of this high dose is unnecessarily selecting for the resistance genotype at greater rates than lower doses, which have been shown to be as effective at managing disease. Thus, regardless of the frequency of resistance to the at-risk single-site fungicide in the fungal population, the lowest dose able to maintain effective disease control should be recommended.

Across the 0%, 5%, and 15% starting resistance ratios, mixing with captan resulted in lower average infection rates numerically in 81% of situations (29 out of 36) when compared to the equivalent dose of fenhexamid alone (Figures 12, 13, 15). However, only four of these situations were statistically significant. In addition, our previous detached fruit assays also found the mixture of captan to be statistically better in control disease severity in many situations. A possible explanation for the small number of situations with statistically significant differences between treatments mixed with captan compared to treatments of the single-site fungicide alone may be attributed to variations between the two growing seasons in the greenhouse. Significantly

higher BBR infection rates were observed in the captan-only fungicide treatment relative to the other treatments in the 2021 season. This difference was not observed in the 2022 season (Data not shown). This may be indicative of ineffective disease control with captan in the 2021 season caused by poor spray coverage on some replicates. In the context of resistance management, between the 5% and 15% starting resistance rates, mixing with captan resulted in lower average resistance rates numerically in 83% of situations (20 out of 24) when compared to the equivalent dose of fenhexamid applied alone (Figures 14, 16). Of these situations, only three were statistically significant. Despite this, a clear trend advocating for the continued use of multi-site fungicides in mixture with at-risk single-site fungicides exists. These findings are consistent with previous modeling studies advocating for the use of multi-site fungicides in mixture with the at-risk single-site fungicides (Hobbelen et al., 2013; Mikaberidze et al., 2014). Further, in context with the significant results discussed in the detached fruit assays, a case can be made in favor of the continuation of tank mixing low-risk, multi-site fungicides like captan. However, the benefits of mixing with captan may not significantly outweigh the prospective input costs of the fungicide when controlling entirely sensitive fungal populations. In our study, the efficacy of fenhexamid for controlling *Botrytis* infection at 0% starting resistance was high regardless of the presence of a mixing partner (Figure 12). Mixing with a multi-site fungicide when spraying an at-risk single-site fungicide in the presence of the resistance genotype is better justified based on our results. Thus, the decision to mix with captan may be predicated on the level or phase of resistance in the field. Screening for resistance to important chemistries is necessary for growers to make the most informed decision for fungicide spray programs. Regardless, a mixture with captan would typically be expected to contribute a net-positive effect on disease control and resistance

management. If the frequency of resistance present in a field is in question, mixing the at-risk fungicide with captan is a safe way to hedge against potential fungicide resistance.

Botrytis infection remains an issue in greenhouse production, beyond grape bunch rot, where resistance to high-risk, single-site mode-of-action fungicides still poses a significant risk. Fungal diseases caused by *Botrytis* spp. are often reported in strawberry, tomato, and ornamental flower greenhouse production as the most destructive (Fan et al., 2016; Rupp et al., 2017; Zhou et al., 2017). Horticultural crops commonly grown in the greenhouse setting are highly susceptible to *Botrytis* infection and chemical control remains the most cost-efficient method of control (Malandrakis et al., 2020). Fenhexamid resistance has been detected in greenhouse production in Greece, China, and Cyprus at the rates of 52%, 2.8%, and 5.9 to 12.7% respectively (Kanetis et al., 2017; Malandrakis et al., 2022; Zhou et al., 2017). Despite this, no recommended fungicide rate for the greenhouse is currently specified for the fungicide Elevate 50 WDG or other similar fenhexamid-based products. To the best of our knowledge, the development of new fungicides and active ingredients are primarily reliant on field trials to determine the labeled rate, which may not be appropriate for greenhouse applications given the drastically different environments between the field and greenhouse settings. Further, the fungicide label explicitly states that the product should never be applied at a rate below the dose listed on the label. This practice may be consistent with the justification for limiting resistance emergence as detailed in previous mathematical modeling studies (van den Bosch et al., 2011). However, it is inconsistent with the best strategies for managing resistance selection once detectable levels of resistant isolates are observed as shown in this study. Our data from the greenhouse assays support the trend of lower doses of the single-site fungicide resulting in lowered resistance selection proving impactful in the validation of previous growth chamber and

modeling studies (Genet et al., 2006; Metcalfe et al., 2000; van den Bosch et al., 2011; van den Bosch, Oliver, et al., 2014). These findings necessitate greater nuance in the language provided on fungicide labels regarding doses for greenhouse application. Given the conclusions from both the detached fruit assays and greenhouse trials, the specification of a recommended, lower, greenhouse application dose may be necessary. A reduced rate specifically labeled for greenhouse application would allow for better resistance management, equivalent or better disease control, lowered exposure for applicators, and lessened chemical inputs for greenhouse producers. Further validation of these fundamental trends is necessary for other pathosystem and active ingredient combinations.

Summary

The development of resistance to the few effective remaining chemical classes of fungicides labeled for control of *Botrytis cinerea* poses a significant risk for crop protection efforts and crop production more broadly. In grapes and other small fruit crops, intensive fungicide use is required for the control of common fungal diseases in open fields, which further contributes to the selection of resistance. In this study, fungicide resistance and disease management strategies regarding fungicide dose, mixtures, and application timing were tested across varied frequencies of resistance to the at-risk fungicide, fenhexamid. These spray tactics were tested in a laboratory environment on detached fruit with validation on whole *Vitis vinifera* 'Cabernet Sauvignon' clusters in the greenhouse setting. Our findings were largely consistent with the previous work of recent mathematical modeling and controlled environment studies. Our results from both the laboratory and greenhouse assays support the lowering of the fungicide application dose of the single-site fungicide for better resistance management. Further, doses significantly lower than the dose recommended on the fungicide label were as effective at controlling disease under these controlled environmental conditions. In addition, our results support the continued use and mixture of multi-site fungicides to limit resistance selection. Surprisingly, fungicide applications after tiny but visible signs of fungal infection were more effective at limiting resistance selection than preventative sprays while providing equivalent disease control. In this pathosystem and active ingredient combination, the most effective resistance management strategies were the application of the at-risk fungicide at the lowest dose capable of effective disease control, mixture with a multi-site fungicide, and delayed fungicide application until first signs of fungal infection.

References

Adaskaveg, J. E., Förster, H., Gubler, W. D., Teviotdale, B. L., & Thompson, D. F. (2005). Reduced-risk fungicides help manage brown rot and other fungal diseases of stone fruit. *California Agriculture*, *59*(2), 109–114. https://doi.org/10.3733/ca.v059n02p109

Alzohairy, S. A., Gillett, J., Saito, S., Naegele, R. N., Xiao, C. L., & Miles, T. D. (2021). Fungicide Resistance Profiles of *Botrytis cinerea* Isolates From Michigan Vineyards and Development of a TaqMan Assay for Detection of Fenhexamid Resistance. *Plant Disease*, *105*(2), 285–294. https://doi.org/10.1094/PDIS-05-20-1087-RE

Amiri, A., & Peres, N. A. (2014). Diversity in the *erg27* Gene of *Botrytis cinerea* Field Isolates from Strawberry Defines Different Levels of Resistance to the Hydroxyanilide Fenhexamid. *Plant Disease*, *98*(8), 1131–1137. https://doi.org/10.1094/PDIS-11-13-1171-RE

Ayer, K. M., Choi, M.-W., Smart, S. T., Moffett, A. E., & Cox, K. D. (2020). The Effects of Succinate Dehydrogenase Inhibitor Fungicide Dose and Mixture on Development of Resistance in Venturia inaequali. *Applied and Environmental Microbiology*, *86*(17), e01196-20. https://doi.org/10.1128/AEM.01196-20

Beckerman, J. L., Sundin, G. W., & Rosenberger, D. A. (2015). Do some IPM concepts contribute to the development of fungicide resistance? Lessons learned from the apple scab pathosystem in the United States. *Pest Management Science*, *71*(3), 331–342. https://doi.org/10.1002/ps.3715

Berg, F., Paveley, N. D., & Bosch, F. (2016). Dose and number of applications that maximize fungicide effective life exemplified by *Zymoseptoria tritici* on wheat – a model analysis. *Plant Pathology*, *65*(8), 1380–1389. https://doi.org/10.1111/ppa.12558

Bettiga, L. J. (2013). *Grape Pest Management, Third Edition*. UCANR Publications.

Brent, K. J. (1995). *Fungicide resistance in crop pathogens: How can it be managed?* GIFAP.

Broome, J. C., English, J. T., Marois, J. J., Latorre, B. A., & Aviles, J. C. (1995). Development of an infection model for Botrytis bunch rot of grapes based on wetness duration and temperature. *Phytopathology®*, *85*(1), 97–102.

Burnett, F. J., & Zziwa, M. C. N. (1997). Effect of application rate on the sensitivity of *Erysiphe graminis* f.sp. *Tritici* to fenpropimorph. *Pesticide Science*, *51*(3), 335–340. https://doi.org/10.1002/(SICI)1096-9063(199711)51:3<335::AID-PS645>3.0.CO;2-Y

Caffi, T., Rossi, V., & Bugiani, R. (2010). Evaluation of a Warning System for Controlling Primary Infections of Grapevine Downy Mildew. *Plant Disease*, *94*(6), 709–716. https://doi.org/10.1094/PDIS-94-6-0709

Chen, M., Brun, F., Raynal, M., & Makowski, D. (2020). Delaying the first grapevine fungicide application reduces exposure on operators by half. *Scientific Reports*, *10*(1), 6404. https://doi.org/10.1038/s41598-020-62954-4

Cosseboom, & Hu, M. (2021). Identification and Characterization of Fungicide Resistance in *Botrytis* Populations from Small Fruit Fields in the Mid-Atlantic United States. *Plant Disease*, *105*(9), 2366–2373. https://doi.org/10.1094/PDIS-03-20-0487-RE

Cosseboom, & Hu, M. (2021). Diversity, Pathogenicity, and Fungicide Sensitivity of Fungal Species Associated with Late-Season Rots of Wine Grape in the Mid-Atlantic United States. *Plant Disease*, *105*(10), 3101–3110. https://doi.org/10.1094/PDIS-01-21-0006-RE

Dean, R., Van Kan, J. A. L., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J. J., Dickman, M., Kahmann, R., Ellis, J., & Foster, G. D. (2012). The Top 10

fungal pathogens in molecular plant pathology: Top 10 fungal pathogens. *Molecular Plant Pathology, 13*(4), 414–430. https://doi.org/10.1111/j.1364-3703.2011.00783.x

Dooley, H., Shaw, M. W., Spink, J., & Kildea, S. (2016). Effect of azole fungicide mixtures, alternations and dose on azole sensitivity in the wheat pathogen *Zymoseptoria tritici*. *Plant Pathology, 65*(1), 124–136. https://doi.org/10.1111/ppa.12395

Dowling, M. E., Hu, M.-J., Schmitz, L. T., Wilson, J. R., & Schnabel, G. (2016). Characterization of *Botrytis cinerea* Isolates from Strawberry with Reduced Sensitivity to Polyoxin D Zinc Salt. *Plant Disease*, *100*(10), 2057–2061. https://doi.org/10.1094/PDIS-02-16-0166-RE

Elad, Y., Pertot, I., Cotes Prado, A. M., & Stewart, A. (2016). Plant Hosts of Botrytis spp. In S. Fillinger & Y. Elad (Eds.), *Botrytis – the Fungus, the Pathogen and its Management in Agricultural Systems* (pp. 413–486). Springer International Publishing. https://doi.org/10.1007/978-3-319-23371-0_20

Elad, Y., Williamson, B., Tudzynski, P., Delen, N., & Elad, Y. (Eds.). (2007). *Botrytis: Biology, pathology and control*. Springer.

Elderfield, J. A. D., Lopez-Ruiz, F. J., van den Bosch, F., & Cunniffe, N. J. (2018). Using Epidemiological Principles to Explain Fungicide Resistance Management Tactics: Why do Mixtures Outperform Alternations? *Phytopathology®*, *108*(7), 803–817. https://doi.org/10.1094/PHYTO-08-17-0277-R

Fan, F., Hamada, M. S., Li, N., Li, G. Q., & Luo, C. X. (2017). Multiple Fungicide Resistance in *Botrytis cinerea* from Greenhouse Strawberries in Hubei Province, China. *Plant Disease*, *101*(4), 601–606. https://doi.org/10.1094/PDIS-09-16-1227-RE

Fan, F., Li, N., Li, G. Q., & Luo, C. X. (2016). Occurrence of Fungicide Resistance in *Botrytis cinerea* from Greenhouse Tomato in Hubei Province, China. *Plant Disease, 100*(12), 2414–2421. https://doi.org/10.1094/PDIS-03-16-0395-RE

Fernández-Ortuño, D., Grabke, A., Bryson, P. K., Amiri, A., Peres, N. A., & Schnabel, G. (2014). Fungicide Resistance Profiles in *Botrytis cinerea* from Strawberry Fields of Seven Southern U.S. States. *Plant Disease, 98*(6), 825–833. https://doi.org/10.1094/PDIS-09-13-0970-RE

Fisher, M. C., Hawkins, N. J., Sanglard, D., & Gurr, S. J. (2018). Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science, 360*(6390), 739–742. https://doi.org/10.1126/science.aap7999

Forster, B., & Staub, T. (1996). Basis for use strategies of anilinopyrimidine and phenylpyrrole fungicides against Z?otrytis. *Crop Protection*, *45*, 529–537.

Genet, J.-L., Jaworska, G., & Deparis, F. (2006). Effect of dose rate and mixtures of fungicides on selection for QoI resistance in populations ofPlasmopara viticola. *Pest Management Science, 62*(2), 188–194. https://doi.org/10.1002/ps.1146

González-Domínguez, E., Caffi, T., Ciliberti, N., & Rossi, V. (2015). A Mechanistic Model of Botrytis cinerea on Grapevines That Includes Weather, Vine Growth Stage, and the Main Infection Pathways. *PLOS ONE, 10*(10), e0140444. https://doi.org/10.1371/journal.pone.0140444

Grabke, A., Fernández-Ortuño, D., & Schnabel, G. (2013). Fenhexamid Resistance in *Botrytis cinerea* from Strawberry Fields in the Carolinas Is Associated with Four Target Gene Mutations. *Plant Disease, 97*(2), 271–276. https://doi.org/10.1094/PDIS-06-12-0587-RE

Gubler, W. D., Ypema, H. L., Ouimette, D. G., & Bettiga, L. J. (1996). Occurrence of Resistance in Uncinula necator to Triadimefon, Myclobutanil, and Fenarimol in California Grapevines. *PLANT DISEASE -ST PAUL-*, *80*(8), 902–909. WorldCat.org.

Hobbelen, P. H. F., Paveley, N. D., Oliver, R. P., & van den Bosch, F. (2013). The Usefulness of Fungicide Mixtures and Alternation for Delaying the Selection for Resistance in Populations of *Mycosphaerella graminicola* on Winter Wheat: A Modeling Analysis. *Phytopathology*®, *103*(7), 690–707. https://doi.org/10.1094/PHYTO-06-12-0142-R

Hu, M.-J., Dowling, M. E., & Schnabel, G. (2018). Genotypic and Phenotypic Variations in *Botrytis* spp. Isolates from Single Strawberry Flowers. *Plant Disease*, *102*(1), 179–184. https://doi.org/10.1094/PDIS-06-17-0891-RE

Hu, M.-J., Fernández-Ortuño, D., & Schnabel, G. (2016). Monitoring Resistance to SDHI Fungicides in *Botrytis cinerea* From Strawberry Fields. *Plant Disease*, *100*(5), 959–965. https://doi.org/10.1094/PDIS-10-15-1210-RE

Kanetis, L., Christodoulou, S., & Iacovides, T. (2017). Fungicide resistance profile and genetic structure of Botrytis cinerea from greenhouse crops in Cyprus. *European Journal of Plant Pathology*, *147*(3), 527–540. https://doi.org/10.1007/s10658-016-1020-9

Keller, M., Viret, O., & Cole, F. M. (2003). *Botrytis cinerea* Infection in Grape Flowers: Defense Reaction, Latency, and Disease Expression. *Phytopathology*®, *93*(3), 316–322. https://doi.org/10.1094/PHYTO.2003.93.3.316

Klittich, C. J. R. (2014). Fungicide Mobility and the Influence of Physical Properties. In K. Myung, N. M. Satchivi, & C. K. Kingston (Eds.), *ACS Symposium Series* (Vol. 1171, pp. 95–109). American Chemical Society. https://doi.org/10.1021/bk-2014-1171.ch005

Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., Gredt, M., & Chapeland, F. (2002). Mechanisms of resistance to fungicides in field strains ofBotrytis cinerea. *Pest Management Science*, *58*(9), 876–888. https://doi.org/10.1002/ps.566

Lucas, J. A., Hawkins, N. J., & Fraaije, B. A. (2015). The Evolution of Fungicide Resistance. In *Advances in Applied Microbiology* (Vol. 90, pp. 29–92). Elsevier. https://doi.org/10.1016/bs.aambs.2014.09.001

MacKenzie, S. J., & Peres, N. A. (2012). Use of Leaf Wetness and Temperature to Time Fungicide Applications to Control Anthracnose Fruit Rot of Strawberry in Florida. *Plant Disease*, *96*(4), 522–528. https://doi.org/10.1094/PDIS-03-11-0181

Malandrakis, A. A., Kavroulakis, N., & Chrysikopoulos, C. V. (2020). Synergy between Cu-NPs and fungicides against Botrytis cinerea. *Science of The Total Environment*, *703*, 135557. https://doi.org/10.1016/j.scitotenv.2019.135557

Malandrakis, A. A., Krasagakis, N., Kavroulakis, N., Ilias, A., Tsagkarakou, A., Vontas, J., & Markakis, E. (2022). Fungicide resistance frequencies of Botrytis cinerea greenhouse isolates and molecular detection of a novel SDHI resistance mutation. *Pesticide Biochemistry and Physiology*, *183*, 105058. https://doi.org/10.1016/j.pestbp.2022.105058

McGrath, M. T. (1996). Successful Management of Powdery Mildew in Pumpkin with Disease Threshold-based Fungicide Programs. *Plant Disease*, *80*(8), 910–916.

Metcalfe, R. J., Shaw, M. W., & Russell, P. E. (2000). The effect of dose and mobility on the strength of selection for DMI fungicide resistance in inoculated field experiments. *Plant Pathology*, *49*(5), 546–557. https://doi.org/10.1046/j.1365-3059.2000.00486.x

Mikaberidze, A., McDonald, B. A., & Bonhoeffer, S. (2014). Can High-Risk Fungicides be Used in Mixtures Without Selecting for Fungicide Resistance? *Phytopathology®*, *104*(4), 324–331. https://doi.org/10.1094/PHYTO-07-13-0204-R

Nair, N., Guilbaud-Oulton, S., Barchia, I., & Emmett, R. (1995). Significance of carry over inoculum, flower infection and latency on the incidence of Botrytis cinerea in berries of grapevines at harvest in New South Wales. *Australian Journal of Experimental Agriculture*, *35*(8), 1177. https://doi.org/10.1071/EA9951177

Northover, J. (1986). Resistance of *Botrytis cinerea* to Benomyl and Iprodione in Vineyards and Greenhouses After Exposure to the Fungicides Alone or Mixed with Captan. *Plant Disease*, *70*(5), 398. https://doi.org/10.1094/PD-70-398

Oxley, S. J. P., Burnett, F., Hunter, E. A., Fraaije, B. A., Cooke, L. R., Mercer, P. C., & Gilchrist, A. (2008). Understanding fungicide mixtures to control Rhynchosporium in barley and minimise resistance shifts. *HGCA, Proj. Rep. 436*, 97.

Petit, A.-N., Vaillant-Gaveau, N., Walker, A.-S., Leroux, P., Baillieul, F., Panon, M.-L., Clément, C., & Fontaine, F. (2010). Determinants of fenhexamid effectiveness against grey mould on grapevine: Respective role of spray timing, fungicide resistance and plant defences. *Crop Protection*, *29*(10), 1162–1167. https://doi.org/10.1016/j.cropro.2010.04.007

Pijls, C. F. N., & Shaw, M. W. (1997). Weak selection by field sprays for flutriafol resistance in *Septoria tritici* . *Plant Pathology*, *46*(2), 247–263. https://doi.org/10.1046/j.1365-3059.1997.d01-227.x

Rupp, S., Weber, R. W. S., Rieger, D., Detzel, P., & Hahn, M. (2017). Spread of Botrytis cinerea Strains with Multiple Fungicide Resistance in German Horticulture. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.02075

Saito, S., Michailides, T. J., & Xiao, C. L. (2016). Fungicide Resistance Profiling in *Botrytis cinerea* Populations from Blueberry in California and Washington and Their Impact on Control of Gray Mold. *Plant Disease*, *100*(10), 2087–2093. https://doi.org/10.1094/PDIS-02-16-0229-RE

Saito, S., Michailides, T. J., & Xiao, C. L. (2019). Fungicide-resistant phenotypes in Botrytis cinerea populations and their impact on control of gray mold on stored table grapes in California. *European Journal of Plant Pathology*, *154*(2), 203–213. https://doi.org/10.1007/s10658-018-01649-z

Simpfendorfer, S. (2016). Evaluation of fungicide timing and preventative application on the control of Fusarium head blight and resulting yield. *Australasian Plant Pathology*, *45*(5), 513–516. https://doi.org/10.1007/s13313-016-0441-4

Steva, H. (1994). Evaluating anti-resistance strategies for control of Uncinula necator. *MONOGRAPHS- BRITISH CROP PROTECTION COUNCIL*, *60*, 59. WorldCat.org.

Thind, T. S., & Hollomon, D. W. (2018). Thiocarbamate fungicides: Reliable tools in resistance management and future outlook: Thiocarbamate fungicides. *Pest Management Science*, *74*(7), 1547–1551. https://doi.org/10.1002/ps.4844

van den Bosch, F., Oliver, R., Berg, F. van den, & Paveley, N. (2014). Governing Principles Can Guide Fungicide-Resistance Management Tactics. *Annual Review of Phytopathology*, *52*(1), 175–195. https://doi.org/10.1146/annurev-phyto-102313-050158

van den Bosch, F., Paveley, N., Shaw, M., Hobbelen, P., & Oliver, R. (2011). The dose rate debate: Does the risk of fungicide resistance increase or decrease with dose?: The dose rate debate. *Plant Pathology, 60*(4), 597–606. https://doi.org/10.1111/j.1365-3059.2011.02439.x

van den Bosch, F., Paveley, N., van den Berg, F., Hobbelen, P., & Oliver, R. (2014). Mixtures as a Fungicide Resistance Management Tactic. *Phytopathology®*, *104*(12), 1264–1273. https://doi.org/10.1094/PHYTO-04-14-0121-RVW

Weber, R. W. S. (2011). Resistance of *Botrytis cinerea* to Multiple Fungicides in Northern German Small-Fruit Production. *Plant Disease*, *95*(10), 1263–1269. https://doi.org/10.1094/PDIS-03-11-0209

Weber, R. W. S., & Hahn, M. (2011). A rapid and simple method for determining fungicide resistance in Botrytis. *Journal of Plant Diseases and Protection*, *118*(1), 17–25. https://doi.org/10.1007/BF03356376

Wilcox, W. F., Gubler, W. D., & Uyemoto, J. K. (Eds.). (2015). *Compendium of Grape Diseases, Disorders, and Pests, Second Edition*. The American Phytopathological Society. https://doi.org/10.1094/9780890544815

Wołejko, E., Łozowicka, B., Kaczyński, P., Jankowska, M., & Piekut, J. (2016). The influence of effective microorganisms (EM) and yeast on the degradation of strobilurins and carboxamides in leafy vegetables monitored by LC-MS/MS and health risk assessment. *Environmental Monitoring and Assessment*, *188*(1), 64. https://doi.org/10.1007/s10661-015-5022-4

Zhou, F., Lu, F., Zhang, C., Qi, H.-X., Wang, X.-D., & Zhang, G.-Z. (2017). Occurrence of fenhexamid resistance in *Botrytis cinerea* from greenhouse strawberries in China. *Journal of Phytopathology*, *165*(7–8), 455–462. https://doi.org/10.1111/jph.12580